NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

Novel insights into the role of nitric oxide and hydrogen sulfide in myocardial protection.

by

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Στη μνήμη των γονιών μου..

Που με έμαθαν ότι σημασία δεν έχει πόσες φορές θα πέσεις,

αλλά πόσες φορές θα καταφέρεις να σηκωθείς.

Ευχαριστίες

"Tell me and I forget..

Teach me and I may remember..

Involve me and I learn.."

Μερικές φορές το λιγότερο που μπορείς να κάνεις είναι να αφιερώσεις λίγο χρόνο και να ευχαριστήσεις ανθρώπους που σου άλλαξαν τη ζωή.

Οι μέντορες δεν βρίσκονται εύκολα.. Είμαι τυχερή που από τα πρώτα μου βήματα στον χώρο της έρευνας ήρθα σε επαφή όχι με έναν αλλά με δύο εξαιρετικούς μέντορες. Είναι δύσκολο να τους διαχωρίσει κανείς. Κάθε ένας τους με την εξέχουσα προσωπικότητα που τον διακρίνει έπαιξαν σημαντικό ρόλο στη διαμορφωσή μου πρώτα σαν άνθρωπο και μετά σαν φοιτήτρια. Με εμπιστεύθηκαν και με καθοδήγησαν όλα αυτά τα χρόνια. Με δίδαξαν πως να δουλεύω ανεξάρτητα, πώς να σκέφτομαι, πώς να επιτυγχάνω τους στόχους μου. Οφείλω ένα πολύ μεγάλο ευχαριστώ και στους δύο συν-επιβλέποντες και «δασκάλους» μου, την κα. Ιωάννα Ανδρεάδου και τον κ. Παπαπετρόπουλο Ανδρέα. Τους ευχαριστώ που μου έδωσαν τη δυνατότητα να δουλέψω δίπλα τους, που διαχειρίστηκαν τη δύσκολη προσωπικότητά μου, που με «έσπρωχναν» όταν εγκατέλειπα και με κρατούσαν όταν υπερέβαλα, που πίστευαν σε εμένα ακόμα και όταν δεν πίστευα στον εαυτό μου. *You both were hard to find.. Difficult to part with (sometimes that was really difficult!!).. but impossible to forget. But that's how only true mentors are.* Εύχομαι να μην σταματήσετε να ζυμώνεται μυαλά και συνειδήσεις και να εμπνέετε νέους ανθρώπους.

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Bibli S-I. : *eNOS phosphorylation by PYK 2 regulates cardioprotection.*

Session: Nitric Oxide (NO)

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PUBLICATIONS:

Selected Posters

1. Andreadou I., Lazari A., **Bibli S-I.**, Gaboriaud-Kolar N., Skaltsounis A.L., Zoga A., Dagres N., Kremastinos D.Th., Anastasiou-Nana M., Iliodromitis E.K. Investigating the role of GSK3beta kinase as a molecular target for myocardial infarct size reduction by using novel pharmacological inhibitors. European Heart Journal (2013) 34 (Abstract Supplement), 158 (Oral presentation)

2. Andreadou I., **Bibli S-I.**, Konstantinou M., Vasileiou S., Gikas E., Lemonakis N., Kremastinos D.Th., Anastasiou-Nana M., Skaltsounis A.L., Iliodromitis E.K., The olive constituent oleuropein triggers a postconditioning-like effect in anesthetized rabbits through GSK3beta inhibition and JAK/STAT activation. European Heart Journal (2013) 34 (Abstract Supplement), 667 (Oral presentation)

3. Andreadou I., **Bibli S-I.**, Zhou Z., Fotopoulou T., Koufaki M., Papapetropoulos A., Iliodromitis E.K., Pharmacological characterization and elucidation of the molecular mechanism of action of a novel purine analogue which has superior effects to ischemic

postconditioning. European Heart Journal (2014) 35 (Abstract Supplement), 386-387 (Oral presentation)

4. **Bibli S-I.**, Andreadou I., Tzimas C., Sanoudou D., Hatzianastasiou A., Kranias E., Kremastinos D.Th., Iliodromitis E.K., Papapetropoulos A., Phospholamban is a final effector of ischemic postconditioning and H_2S induced cardioprotection. European Heart Journal (2014) 35 (Abstract Supplement), 387 (Oral presentation)

5. Andreadou I., Mastromanolis E., **Bibli S-I.**, Zoga A., Dagres N., Kremastinos D.Th., Anastasiou-Nana M., Iliodromiths E.K., Remote perconditioning limits the infarct size in anaesthetized rabbits after stimulation of STAT5, independently of the RISK and SAFE pathways. European Heart Journal (2013) 34 (Abstract Supplement), 340 (Moderate poster)

6. Andreadou I., Provokas E., Zoga A., **Bibli S-I.**, Sigala F., Anastasiou-Nana M., Kremastinos D.Th., Iliodromitis E.K., Acute administration of pravastatin in contrast to simvastatin and postconditiong reduces infarct size in hyperlipidemic rabbits through activation of endothelial nitric oxide synthase. European Heart Journal (2011) 32 (Abstract Supplement), 364

7. Andreadou I., **Bibli S-I.**, Zhou Z., Zoga A., Kremastinos D.Th., Anastasiou-Nana M., Iliodromitis E.K., Papapetropoulos A.. Hydrogen sulfide induces pharmacological postconditioning in anesthetized rabbits through PKG activation. European Heart Journal (2011) 32 (Abstract Supplement), 576

8. **Bibli S-I.**, Andreadou I., Zhou Z., Zoga A., Kremastinos D.Th., Anastasiou-Nana M., Iliodromitis E.K., Papapetropoulos A., Exogenous hydrogen sulfide induces pharmacological postconditioning in anesthetized rabbits through AKT/PKG/PLB activation, independently of mitoKATP channels opening and JAK/STAT pathway. European Heart Journal (2013) 34 (Abstract Supplement), 606-607

9. Andreadou I., **Bibli S-I.**, Efentakis P., Vasileiou S., Pouli N., Zoga A., Anastasiou-Nana M., Kremastinos D.Th., Iliodromitis E.K., Ranolazine triggers pharmacological preconditioning and postconditioning in anesthetized rabbits through activation of RISK pathway. European Heart Journal (2013) 34 (Abstract Supplement), 1020

10. **Bibli S-I.**, Andreadou I., Zoga A., Vasileiou S., Anastasiou-Nana M., Kremastinos D.Th., Iliodromitis E.K., Papapetropoulos A., Thioglycine, a slow-releasing H2S donor, exerts cardioprotection in anesthetized rabbits through activation of Akt kinase. European Heart Journal (2013) 34 (Abstract Supplement), 1021

11. Andreadou I., Flevari P., Leftheriotis D., Vasileiou S., Zoga A., **Bibli S-I.**, Anastasiou-Nana M., Iliodromitis E.K.. The effect of beta adrenergic and muscarinic receptors blockade on perconditioning in anesthetized rabbits. European Heart Journal (2014) 35 (Abstract Supplement), 306

12. **Bibli S-I.**, Glynos C., Andreadou I., Hatzianastasiou A., Iliodromitis E.K., Papapetropoulos A., Cigarette smoking negates the beneficial effect of ischemic postconditioning in limiting the infarct size through insufficient activation of AKT and eNOS. European Heart Journal (2014) 35 (Abstract Supplement), 307-308

13. **Bibli S-I.**, Andreadou I., Sanoudou D., Chatzianastasiou A., Kranias E., Iliodromitis E.K., Papapetropoulos A., Investigating the role of phospholamban as end point of ischemic

postconditioning and H2S-induced cardioprotection. Basic Clinc Pharmacol & Toxicl (2014) 115(Suppl1):34

14. **Bibli S-I**., Papapetropoulos A., Daiber A., Steven S., Brouckaert P., Iliodromitis EK., Andreadou I., Endothelial nitric oxide synthase activation as a novel mechanism for nitroglycerin-induced cardioprotection. ECS 2015, London (Moderate poster)

15. Andreadou I., **Bibli S-I.**, Zoga A., Farmakis D., Efentakis P., Mastromanolis E., Kremastinos DTh., Iliodromitis EK., Investigating the molecular signaling pathway of Perconditioning: focused on STAT5 and eNOS inhibition. ECS 2015, London

16. Glynos C., Bibli S-I., Pavlidou A., Katsaounou P., Kalomenidis I., Zakynthinos S.,Papapetropoulos A., Comparison of the effects of e-cigarette vapor vs cigarette smoke on lung function and inflammation in mice. European Respiratory Journal Sep 2015, 46 (suppl 59)

17. **Bibli S-I.,** Zhou Z., Zukunft S., Fissthaler B., Andreadou I., Szabo C., Fleming I., Papapetropoulos A. Tyrosine phosphorylation of eNOS regulates myocardial survival after an ischemic insult: role of PYK2. Submitted ESC 2016.

Peer-reviewed journals

Research Papers

1. **Bibli S-I.**, Toli E.V., Vilaeti AD., Varnavas V.C., Baltogiannis G.G., Papalois A., Kyriakides Z.S., and Kolettis T.M., Endothelin-B receptors and left ventricular dysfunction after regional versus global ischaemia-reperfusion in rat hearts. Cardiovascular Research and Practice, 2012;2012:986813

2. Koufaki M., Fotopoulou T., Iliodromitis E.K., **Bibli S-I.**, Zoga A., Kremastinos D.T., Andreadou I., Discovery of 6-[4-(6-nitroxyhexanoyl)piperazin-1-yl)]-9H-purine, as pharmacological post-conditioning agent. Bioorg Med Chem. 2012; 20(19):5948-56. (IF: 2.921).

3. Iliodromitis K.E., Farmakis D., Andreadou I., Zoga A., **Bibli S-I.**, Manolaki T., Dagres N., Iliodromitis E.K., Anastasiou-Nana M., Kremastinos D.Th., Various models of cardiac conditioning in single or sequential periods of ischemia: comparative effects on infarct size and intracellular signalling. Int J Cardiol 2012; S0167-5273(12)01642-7 (IF: 7.078).

4. **Bibli S-I.**, Andreadou I., Lazaris E., Zoga A., Varnavas V., Andreou C.C., Dagres N., Iliodromitis E.K., Kyriakides Z.S., Myocardial protection provided by chronic skeletal muscle ischemia is not further enchanced by ischemic pre- or postconditioning: Comparative effects on intracellular signaling. J Cardiovasc Pharmacol Ther. 2014;19(2):220-7. (IF:2.380)

5. Andreadou I., Mikros E., Ioannidis K., Sigala F., Naka K., Kostidis S., Farmakis D., Tenta R., Kavantzas N., **Bibli S-I.**, Gikas E., Skaltsounis L., Kremastinos D.Th., Iliodromitis E.K., Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. J Mol Cell Cardiol. 2014;69:4-16..(IF:5.034)

6. **Bibli S-I.**, Iliodromitis E.K., Lambertucci C., Zoga A., Lougiakis N., Dagres N., Volpini R., Dal Ben D., Kremastinos D.Th., Tsantili Kakoulidou A., Cristalli G., Andreadou

I., Pharmacological postconditioning of the rabbit heart with non-selective, A1 , A2A and A3 adenosine receptor agonists. J Pharm Pharmacol. 2014;66(8):1140-9. (IF:.2.16)

7. Andreadou I., Benaki D., Efentakis P., **Bibli S-I.**, Milioni AI., Papachristodoulou A., Zoga A., Skaltsounis AL., Mikros E., Iliodromitis EK., The natural olive oil constituent oleuropein induces nutritional cardioprotectionin normal and cholesterol fed rabbits: comparison with preconditioning. Planta Med. 2015;81(8):655-63(IF:.2.15)

8. Andreadou I., **Bibli S-I.**, Mastromanolis E., Zoga A., Efentakis P., Farmakis D., Kremastinos D.Th., Iliodromitis E.K., Transient carotid ischemia as a remote conditioning stimulus for myocardial protection in anesthetized rabbits: Insights into intracellular signaling. Int J Cardiol. 2015 Feb 7;184C:140-151.(IF:6.175)

9. **Bibli S-I.**, Yang G., Zhou Z., Wang R., Topouzis S., Papapetropoulos A., Role of cGMP in hydrogen sulfide signaling. (review article)Nitric Oxide. 2015 Apr 30;46:7-13.(IF:3.521)

10. **Bibli S-I**., Andreadou I., Chatzianastasiou A., Tzimas C., Sanoudou D., Kranias E., Brouckaert P., Coletta C., Szabo C., Kremastinos DT., Iliodromitis EK.,Papapetropoulos A.,Cardioprotection by H2S engages a cGMP-dependent protein kinase G/phospholamban pathway. Cardiovascular Research. 2015;106(3):432-42. (IF:5.992)

Under revision

Bibli S-I., Andreadou I., Glynos K., Chatzianastasiou A., Zakynthinos S., Iliodromitis EK., Papapetropoulos A., Exposure to cigarette smoke abrogates the beneficial effects of ischemic postconditioning. Am J Physiol Heart Circ Physiol

Submitted

Bibli S-I., Zhou Z., Andreadou I., Zukunft S., Fissthaler B., Brouckaert P., Iliodromotis EK., Fleming I., Papapetropoulos A., Tyrosine phosphorylation of eNOS regulates myocardial survival after an ischemic insult: role of PYK2. Cardiovascular Research

Bibli S-I., Papapetropoulos A., Daiber A., Steven S., Kypreos K., Brouckaert P., Iliodromitis EK., Andreadou I., Paradoxical requirement of eNOS in nitroglycerin-induced cardioprotection. Circulation

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Abstract

Background: Ischemia (I) and reperfusion (R) induced organ injury contributes to morbidity and mortality in pathologic conditions including myocardial infarction. The initial tissue damage inflicted by I is paradoxically aggravated during R leading to elevated myocardial infarction. In this context the ischemic preconditioning (PreC) and postconditioning (PostC) are powerful endogenous mechanisms that limit myocardial infarct size. However, although the great knowledge on the molecular mechanisms underlying conditioning phenomena, translation into clinical practice has not been an easy task. Translation of conditioning has been unsuccessful is that patients suffering from acute myocardial infarction in the presence of conditions and/or comorbidities. Since the therapeutic benefit of mechanical PostC (the most clinical relevant) has been challenged, administration of pharmacological agents that act as PostC mimetics might be a preferred approach. In this context, the gaseous transmitters - NO and H_2S- have been shown both to play a predominant role in the pathophysiology of I-R injury and confer cardioprotection as conditioning mimetics. Thus, a better understanding of i) the pathopshysiology of myocardial I-R injury; ii) the underlying mechanisms of signal transduction pathways in conditioning phenomena and iii) the molecular targets of the conditioning mimetics as NO and H_2S; may lead to the establishment of novel and clinically applied therapeutic approaches.

Aim: In the present thesis, we sought to evaluate the role and the underlying molecular mechanisms of endogenous produced or exogenous administrated gasotransmitters, NO and H_2S, on i) the pathophysiology of myocardial I-R injury; in which we sought to correlate the endogenous production of NO by eNOS and its phosphorylation status with cardiomyocyte survival and to investigate the contribution of the novel kinase PYK2 in the regulation of eNOS during myocardial infarction; ii) PostC mimicking effects in myocardial infarction and; in which we sought to determine whether acute low dose NTG (as an NO donor) and NaHS (as an H_2S donor) exert direct effects on myocardial infarct size and further compare the underlying signaling of the two gaseous transmitters in the context of cGMP related pathways and iii) the physiological role of these two gaseous molecules in cigarette smoking; in which we investigated the effects of cigarette smoke (CS) exposure on intracellular myocardial signaling, infarct size after I-R and the potential interference with ischemic conditioning. The aims were assessed by performing four separate experimental studies.

Methods and Results: In the first study oxidative stress injury by H_2O_2 led to an increase of PYK2 phosphorylation on its activator site (Y402) and an increase in eNOS phosphorylation on both the inhibitory site Y656 and on the activator site S1176 in H9c2 cardiomyocytes. Both H_2O_2–induced phosphorylation events were abolished by pharmacological inhibition or gene knockdown of PYK2. Activity assays demonstrated that Y657 exerts a dominant effect over S1176. In cell viability experiments, survival was augmented under either oxidative stress or oxygen-glucose deprivation injury when PYK2 was inhibited; this effect was reversed by inhibition of NO production. Our findings were confirmed *in vivo*; I-R activated PYK2 early during R, leading to eNOS phosphorylation on Y656 and reduced NO output, as judged by the low tissue cGMP levels. Moreover, pharmacological blockade of PYK2 alleviated eNOS inhibition and prevented cardiac damage following I-R in wild-type, but not in eNOS KO mice.

In the second and third study low dose IV NTG and NaHS reduced infarct size in anesthetized male rabbits without affecting haemodynamics. However differences were detected in the downstream pathways. On one hand co-administration of PI3K or NOS inhibitors along with

NTG abrogated NTG's beneficial effect. Inhibition of iNOS, did not affect the cardioprotection afforded by NTG whilst inhibition of nNOS increased NTG's benefit. To further evaluate the role of eNOS in NTG induced cardioprotection, we treated eNOS KO mice with NTG; NTG had no effect on infarct size in eNOS KO mice, although its cardioprotective actions were reproduced in the murine wild type heart but not in the wild type ones with nitrate tolerance. In addition, eNOS and AKT phosphorylation were higher in NTG-treated rabbits compared to the control group. However, no phosphorylation of eNOS was detected in the murine hearts. On the other hand NOS inhibition did not alter the effect of NaHS in rabbit hearts. Unlike what was observed in rabbits, genetic or pharmacological inhibition of eNOS abolished the infarct-limiting effect of NaHS in mice. The signaling differences continued downstream as NTG administration failed to increase cardiac cGMP levels while the PKG inhibitor DT-2 failed to reverse NTG's infarct limiting effects, whilst NaHS increased cardiac cGMP levels and DT-2 abrogated the protective effects of NaHS, an effect not observed by addition of the control peptide TAT. Further studies revealed that each gaseous molecule follows different downstream signaling. Mechanistically, NTG protected through a CypD-dependent manner as NTG failed to confer additional protection in hearts from CypD KO mice, which already exhibited smaller infarct size than their wild type littermates. In addition, NTG offered cardioprotection in ApoE KO mice indicating that it could be a potent cardioprotective agent in the presence of endothelial dysfunction conditions. Interestingly, NTG effects were mediated due to an eNOS mediated nitro-oxidative stress reduction in the ischemic myocardium. As for NaHS, the K_{ATP} channel inhibitor glibenclamide partially reversed the effects of NaHS, while inhibition of mitochondrial K_{ATP} did not modify the NaHS response. NaHS enhanced phosphorylation of phospholamban (PLN), in a PKG-dependent manner. To further investigate the role of PLN in H_2S-mediated cardioprotection, wild type and PLN KO mice underwent I-R. NaHS did not exert cardioprotection in PLN KO mice.

In the fourth study exposure of mice to CS increased blood pressure, caused cardiac hypertrophy, up regulated the NOS/sGC/cGMP pathway whilst decreased H_2S generating enzymes expression.. Exposure to CS did not increase the infarction compared to the room air (RA)-exposed group. PreC application was beneficial for both CS and RA vs non-conditioned animals. PostC application was effective only in RA animals, whilst the infarct size-limiting effect was not preserved in the CS group. Differences in oxidative stress markers, H_2S generating enzymes, Akt and eNOS phosphorylation and cGMP levels were observed between RA and CS groups. The beneficial effect of ischemic PreC is preserved in mice exposed to CS, as it does not affect the cardioprotective signaling; in contrast to PostC.

Conclusions: The conclusions of the present thesis can summarized as follows i) PYK2 is a pivotal regulator of eNOS function in the pathophysiology of myocardial infarction and could serve as a new cardioprotective strategy; ii) Exogenous administration of NO and H_2S donors induces pharmacological postconditioning and reduces myocardial infarct size. The two gaseous molecules act different in the upstream pathways, although both either directly (acting in a CypD dependent manner; the case of NTG) or indirectly (acting due to a cGMP/PKG/PLN pathway; the case of NaHS) preserve mitochondrial integrity and iii) CS is a new comorbidity that blunts PostC beneficial effects in the myocardium. PostC fails to protect CS-exposed mice due to impaired activation of the Akt/eNOS/cGMP axis that occurs in parallel to enhanced oxidative stress

General Introduction

Part I: Pathophysiology of myocardial ischemia reperfusion injury.

1. Ischemia-Reperfusion injury

1.1. Definition

Ischemia; is a condition where the blood flow, and therefore oxygen, is restricted to a part of the body. Reduced blood flow to an organ is usually caused due to a constricted or blocked artery. Ischemia leads to cell death (necrosis and apoptosis) due to oxygen and nutrients insufficiency. Specifically, myocardial ischemia refers to lack of coronary blood flow and oxygen to the heart muscle[1].

Reperfusion; is the restoration of the blood flow to the part of the previously ischemic organ.

1.2. Basic Mechanisms of Cell Death

For many years, ischemia reperfusion-induced cell death was thought to occur by extrinsic factors, such as loss of energy supply, elaboration of inflammatory mediators and toxic molecules, and mechanical injury, a mode of cell death termed necrosis (oncosis). However, it is now recognized that cells can also be programmed to die by cellular signaling mechanisms via the processes of apoptosis and autophagy[2]. In addition, an emerging body of evidence indicates that the apparently random and uncontrolled events associated with necrosis may, under certain circumstances, actually involve the mobilization and coordination of specific signaling mechanisms in a fourth death pathway termed programmed necrosis or necroptosis[3].

1.2.1. Apoptosis

Apoptotic mechanisms are divided into the "extrinsic" and "intrinsic" pathways, although there is considerable cross talk between the two pathways[4, 5]. The "extrinsic" pathway involves the activation and subsequent trimerization of receptors as Fas, TNFα and TRAIL, which, in turn, recruits a number of death domain-containing proteins such as FADD and TRADD to the receptor complex. This death-inducing signaling complex activates the protease caspase-8, which, in turn, cleaves and activates caspase-3. Caspase-3 acts as the cell's executioner by proteolyzing many cellular proteins[4, 5].

Regarding the "intrinsic" pathway, cytotoxic stimuli as ischemia-reperfusion injury induce the translocation and integration of pro-death members of the Bcl2 protein family (e.g., Bax, Bak) into the outer mitochondrial membrane[4, 5]. These proteins, by a mechanism that still remains controversial, permeabilize the outer membrane, thereby enabling the release of pro-apoptotic proteins from the inter membrane space, most notably cytochrome *c*, Smac/DIABLO, Omi/HtrA2, and endonuclease-G (endoG) and form the resultant "apoptosome". Caspases activation or DNA fragmentation follows resulting in cell death[4, 5]. Apoptotic mechanism of cell death is briefly described in Figure 1.

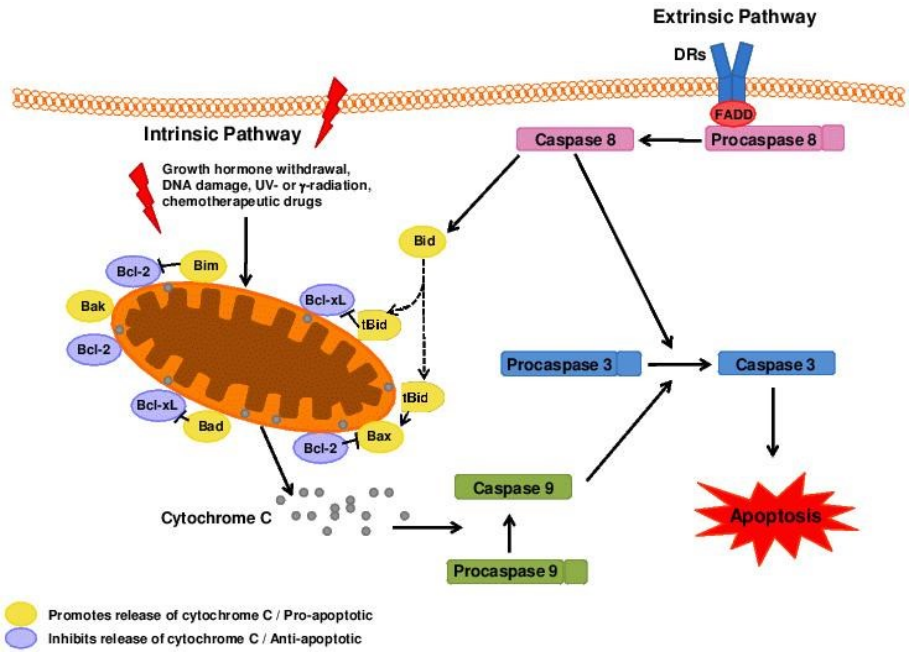


Figure 1: Apoptosis is critical for cardiac survival. It can be mediated by the mitochondrial dependent intrinsic pathway and by the death receptor mediated extrinsic pathway. DRs: death receptors, FADD: Fas-Associated protein with Death Domain[6].

1.2.2. Autophagy

Autophagy is the cell's main mechanism for disposal of obsolete or damaged organelles and protein aggregates, thereby providing a "housekeeping" function. It is also the survival mechanism of cells to withstand stressful conditions, such as starvation, hypoxia, mitochondrial dysfunction, and infection by generating amino acids and fatty acids for maintenance of cell function, or by removing intracellular pathogens. However, uncontrolled autophagy will ultimately lead cell death and may contribute to ischemia- reperfusion injury.

Autophagy is tightly regulated and is mediated by specific pathways. The main controller is mammalian target of rapamycin (mTOR), which inhibits autophagy. However, under conditions of nutrient withdrawal or stress, mTOR is inactivated[7-9]. This depresses other kinases (Atg1, Atg13 and Atg17) which initiate formation of the phagophore. Formation of the phagophore is further facilitated by another complex which, in turn, recruits Atg12, Atg5, and Atg8 (also called LC3), which are essential for the elongation of the membrane and completion of the autophagosome leading to cell death[7-9]. Cardiomyocyte autophagy mechanisms are briefly described in Figure 2.

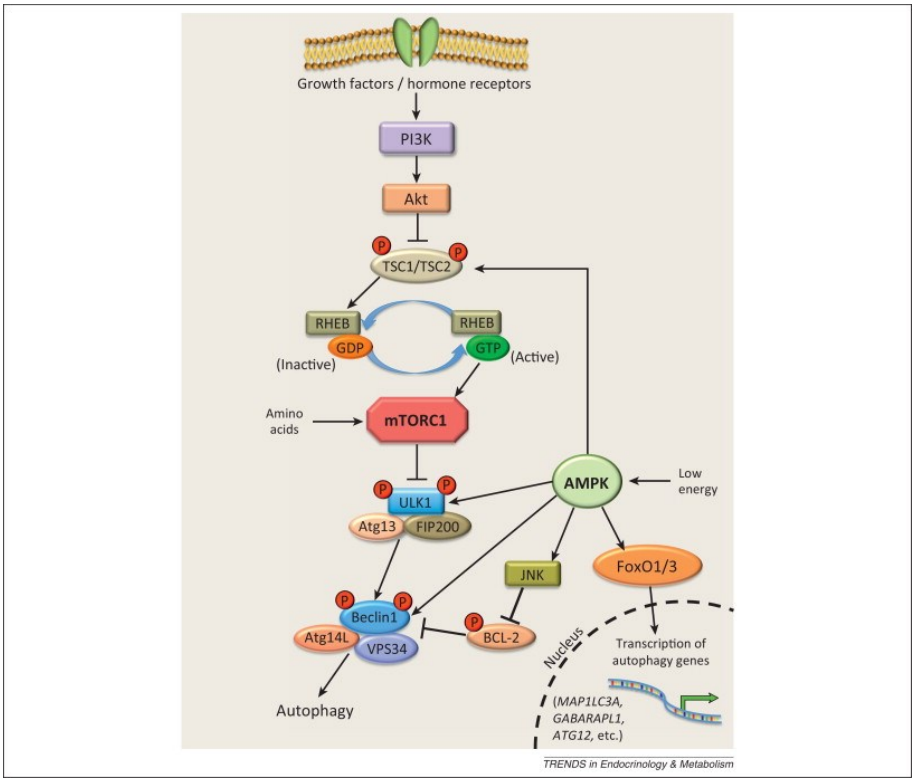


Figure 2: Cardiomyocyte autophagy. Regulation of autophagy by mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). Insulin and growth factors signal through phosphoinositide 3-kinase (PI3K) and Akt to inhibit the tuberous sclerosis (TSC)1/2 complex. Inhibition of this complex allows active Ras homolog enriched in brain (RHEB)–GTP to activate mTOR in the mTORC1 complex, leading to phosphorylation (P) of the ULK1 (unc-51-like autophagy activating kinase 1) complex and inhibition of autophagy. Amino acids can also activate the mTORC1 complex by a separate mechanism. AMPK responds to low cellular energy levels by activating ULK1 via phosphorylation, resulting in activation of autophagy. Additionally, AMPK can initiate autophagy by disrupting BCL-2 inhibition of Beclin 1, by activating forkhead box protein O (FoxO) transcription factors, and by directly activating TSC1/2 and Beclin 1.[10]

1.2.3. Necrosis and necroptosis

Necrosis is characterized morphologically by swelling of cells and their constituent organelles, mitochondrial dysfunction, lack of nuclear fragmentation, plasma membrane rupture, and leakage of intracellular contents. In contrast to the programmed nature of apoptosis and autophagy, necrosis was believed to occur by random, uncontrolled processes that lead to the "accidental" death of the cell in response to overwhelming stress. However, the concept of programmed necrosis, also termed necroptosis, especially under conditions like ischemia-reperfusion, is gaining acceptance. It is now known that cell stress or death receptor activation mobilizes and activates a group of serine/threonine kinases called receptor interacting proteins (RIPs). In particular, RIP1 and RIP3 appear to act in coordination as mediators of necrosis[3, 11, 12]. Activation of RIPs 1 and 3, in turn, leads to increased ROS production either through activation of NADPH oxidases[13], or increased mitochondrial

oxidant production[12], depending on the cell type. The finding that necrostatin-1 (a small tryptophan-based compound that inhibits cell death invoked by TNFα) reduces TNFα-induced necrotic cell death through inhibition of RIP1 kinase activity supports the concept of receptor-induced necrosis via a controlled cellular process[11].

One potential mitochondrial target for RIP-mediated necrosis is the mitochondrial permeability transition (mPT) pore (P). The mPTP is a large, nonspecific channel in the inner mitochondrial membrane that is opened in response to excessive production of ROS and to Ca^{2+} overload of the mitochondrial matrix[4, 14-16], both of the above occur during ischemia and more specifically in the early minutes of reperfusion. This sudden increase in inner membrane permeability dissipates the proton electrochemical gradient ($\Delta\psi_m$), leading to ATP depletion, further ROS production, and ultimately swelling and rupture of the organelle. Although originally proposed as a mediator of apoptosis, recent genetic studies have suggested that the mPTP opening (Figure 3) is predominantly involved in necrosis[4, 14-16].

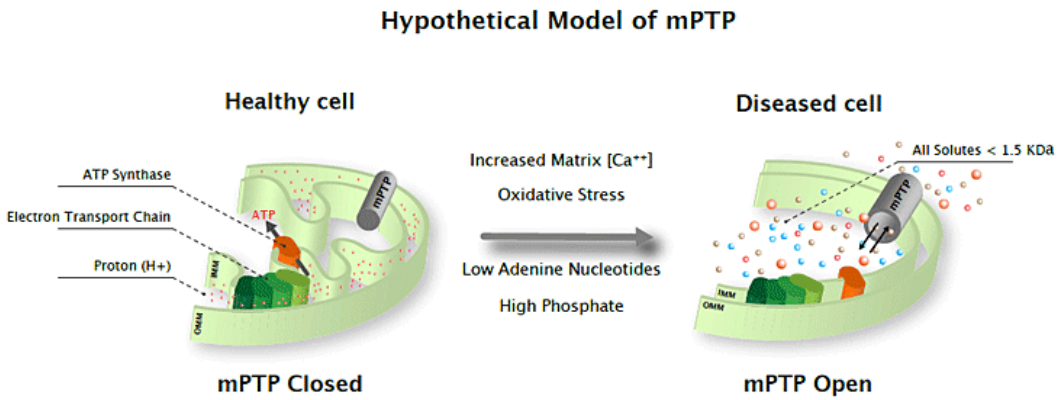


Figure 3: mPTP opening. Various factors, including calcium overload and oxidative stress, open, in the inner mitochondrial membrane, the mitochondrial permeability transition pore (mPTP). This uncouples oxidative phosphorylation and compromises intracellulare ATP levels eventually leading to necrotic cell death[16].

In conclusion myocardial injury after ischemia-reperfusion is modulated by at least two morphologically distinct pathways, namely, necrosis and apoptosis. Both necrosis and apoptosis contribute to increased myocardial infarct size and, depending on their relative contribution, act to modulate post-ischemic functional recovery. However, recent evidence suggest that inhibition of caspase activity in early reperfusion and therefore inhibition of apoptotic cell death does not improve immediate post-ischemic functional recovery (Unpublished data from ECS summer school 2015, Nice, France), questioning the role of apoptosis in myocardial ischemia-reperfusion injury.

1.3. General mechanisms underlying Ischemia-Reperfusion injury

The mechanisms contributing to the pathogenesis of ischemia-reperfusion injury are multifactorial, complex, and highly integrated, with the net result of the perturbations induced by ischemia and invoked when the blood is resupplied causing damage to all biomolecules in cells and tissues.

Ischemia-reperfusion injury is mainly characterized by the following:

(1) ATP depletion; as a result of oxygen depletion which further causes a switch from aerobic to anaerobic metabolism

(2) Ions homeostasis imbalance; the effect of ion ATPase exchangers dysfunction

(3) pH oscillations-pH paradox; starting with the orchestrator lactate and further leading to H^+ concentration impairment

(4) Ca^{2+} paradox; occurring especially during the early minutes of reperfusion

(5) Oxygen paradox; generation of reactive oxygen (ROS) and nitrogen species (RNOS) that is fueled by re-introduction of molecular oxygen when the blood flow is re-established and

(6) mitochondrial dysfunction; which occurs due to opening of the mitochondrial permeability transition (mPT) pore[17] . Described in brief in Figure 4.

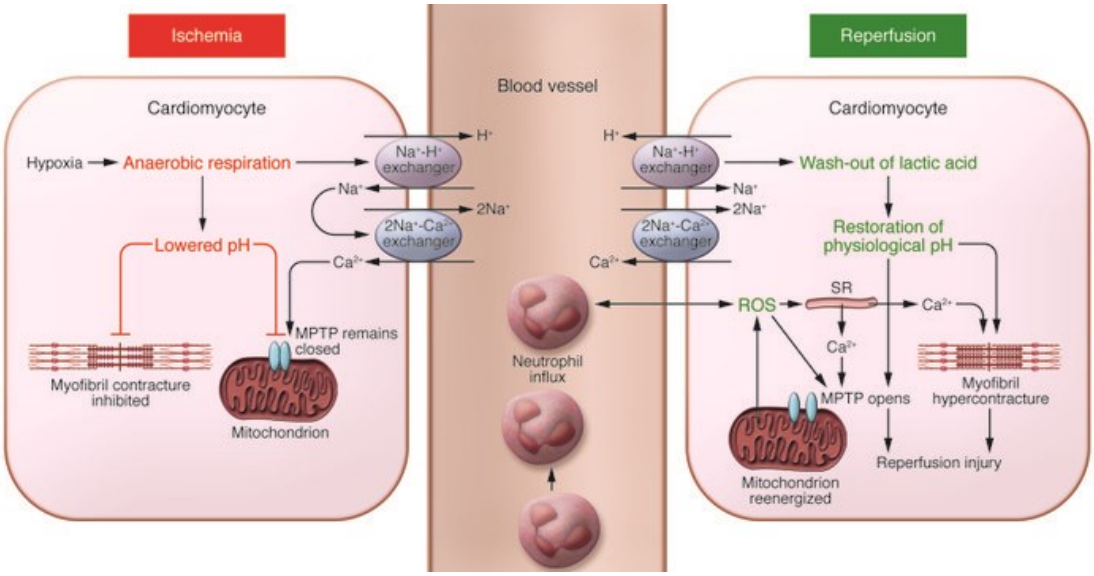


Figure 4: Schematic illustration of the main proponents of acute myocardial ischemia/reperfusion injury. Putative mechanisms of the calcium and free radical paradox in the generation of ischemia/reperfusion injury[18].

In brief, in its classic manifestation, occlusion of an arterial bed limits blood supply and results in a severe insufficiency of oxygen and nutrients, causing organ-tissue hypoxia. The deprivation of oxygen and nutrient supply results in a series of abrupt biochemical and metabolic changes within the myocardium. The absence of oxygen halts oxidative

phosphorylation, leading to mitochondrial membrane depolarization, ATP depletion, and inhibition of myocardial contractile function. This process is exacerbated by the breakdown of any available ATP, as the F1F0 ATPase functions in reverse to maintain the mitochondrial membrane potential, resulting in ATP hydrolysis and an increase in mitochondrial inorganic phosphate[17, 18]. During acute ischemia the absence of oxygen switches cellular metabolism to anaerobic respiration, resulting in the production of lactate and a drop in intracellular pH. To buffer this accumulation of hydrogen ions the Na^+-H^+ exchanger is activated, to extrude H^+ from the cell in exchange for Na^+ overload[19, 20]. Na^+ entry further activates the Na^+-Ca^{2+} exchanger to function in reverse to extrude Na^+ and leads to intracellular Ca^{2+} overload[21]. Cellular ATP depletion ceases function of Na^+-K^+ ATPase, thereby exacerbating intracellular Na^+ overload. The acidic conditions during ischemia prevent the opening of the mitochondrial permeability transition pore (mPTP) and cardiomyocyte hypercontracture at this time. However, the changes in ion homeostasis are accompanied by opening of the mPTP, which dissipates mitochondrial membrane potential and further impairs ATP production[22]. In myocardium, the molecular changes described above during ischemia are accompanied by activation of intracellular proteases (calpains) which damage myofibrils and produce hypercontracture and contracture band necrosis. The degree of tissue injury varies in extent with the magnitude of decrease in the blood supply and with the duration of the ischemic period. Although the absence of O_2 should reflect to minimum ROS production during reperfusion, however ROS are formed either by oxygen deprived mitochondria or from the endothelium.

Although prompt reperfusion restores the delivery of oxygen and substrates required for aerobic ATP generation and normalizes extracellular pH by washing out accumulated H^+, reperfusion itself appears to have detrimental consequences. During reperfusion, the electron transport chain is reactivated, generating ROS. Other sources of ROS include xanthine oxidase (endothelial cells) and NADPH oxidase (neutrophils). ROS mediate myocardial reperfusion injury by inducing the opening of the mPTP, acting as a neutrophil chemoattractant, and mediating dysfunction of the sarcoplasmic reticulum (SR). This contributes to intracellular Ca^{2+} overload and damages the cell membrane by lipid peroxidation, inducing enzyme denaturation and causing direct oxidative damage to DNA. Reperfusion and reactivation of the Na^+-H^+ exchanger result in washout of lactic acid, resulting in the rapid restoration of physiological pH. The restoration of the mitochondrial membrane potential drives calcium into the mitochondria, which can also induce mPTP opening[18, 23].

1.4. Gasotransmitters as signaling molecules in ischemia-reperfusion induced cell death: The role of endogenous produced NO and H_2S.

1.4.1. Gasotransmitters definition:

Gasotransmitters (gaseous transmitters), a newly characterized class of molecules is classified as follows:

i) They are small molecules of gas, like nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S).

ii) They are freely permeable to membrane. As such, their effects will not rely on cognate membrane receptors.

iii) They are endogenously and enzymatically generated and their generation is regulated.

iv) They have well-defined specific functions at physiologically relevant concentrations.

v) Their cellular effects may or may not be mediated by second messengers, but should have specific cellular and molecular targets[24].

1.4.2. The role of H_2S in the pathophysiology of ischemia-reperfusion injury.

H_2S is generated from endogenous sources and is physiologically present in different tissues. Endogenous H_2S generating enzymes have been identified in mammals. De-sulfhydration of cysteine is the major source of H_2S in mammals and is catalyzed by the trans-*sulfuration* pathway enzymes cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3- MST). Cystathionine can be converted by CSE to form H_2S. CBS can form cystathionine from serine and homocysteine, and additionally can form H_2S from cysteine. Cysteine, along with alpha-ketoglutarate (alpha- KG), is converted into 3- mercaptopyruvate (3MP) by cystine aminotransferase (CAT). 3MP can then be broken down by 3MST to form H_2S[25]. In the heart, CBS and 3MST seem not to play any significant role in generating H_2S under normal conditions, but CSE appears to be involved in the endogenous generation of H_2S[26, 27]. The observation that the heart contains significant H_2S synthesizing enzyme suggests that it represents an important source of H_2S generation[28] .

The understanding of the regulation of H_2S levels and CSE in the pathophysiology process of ischemia-reperfusion injury is still in its infancy. However, as post-translation modifications of CSE have not been yet reported in the literature in the context of myocardial ischemia-reperfusion injury, the data that suggest an important role of H_2S in ischemia-reperfusion injury are driven through CSE activity studies and functional studies which evaluate the size of myocardial infarction upon CSE inhibition or knock down[27, 29-35].

Although, in general the cytoprotection afforded by H_2S is associated with up-regulation of antioxidant pathways[36], inhibition of myocardial inflammation[37], preservation of mitochondrial structure and function[37] after ischemia and inhibition of apoptosis[37, 38]; however, there is only one study, described below that explains how endogenous H_2S confers cardioprotection.

1.4.2.1. CSE activity studies during ischemia-reperfusion injury.

In 2007 Chuah et al[29]., evaluated by Stipank & Beck method (Methylene blue) the activity of CSE under ischemia-reperfusion injury in the left ventricle. They found that CSE is the enzyme responsible for hydrogen sulfide production in the myocardium. In addition, the authors of this study, by using a selective inhibitor of CSE (PAG) [39]during acute myocardial infarction in a rat in vivo model of myocardial infarction reported that both expression and activity of CSE were reduced[29]. Later on, it was reported that in primary neonatal rat cardiomyocytes, CSE expression was remarkably suppressed after hypoxia treatment[31]. Recently these findings were replicated in cardiac stem cells as it was reported that CSE expression and activity was inhibited by hypoxia/serum deprivation treatment in these cells. In addition, functional effects on apoptotic death were reported, as, overexpression of CSE markedly prevented death, whilst CSE inhibitors reversed and exacerbated apoptotic death[30].

However, we have to keep in mind that a direct and reliable method measuring CSE activity, except methylene blue, has not been described yet.

1.4.2.2. Lessons from CSE inhibition studies and knockout models on the involvement of H_2S in ischemia-reperfusion injury

It has been reported that inhibition of CSE enzyme by administration of PAG increases myocardial infarct size in in vivo pig[34] and rat[27, 35] models of myocardial ischemia-reperfusion injury. These studies were the first suggesting that the endogenous H_2S is produced during myocardial ischemia in sufficient amounts to limit infarct size.

However, as although PAG is a potent inhibitor of CSE[39], the use of a pharmacological inhibitor –with many off target effects- as the only tool to evaluate the role of CSE during myocardial infarction needs further evaluation.

More recent and sophisticated studies strengthened the cardioprotective role of endogenous H_2S. Calvert et al., by using a CSE cardiac-restricted overexpression murine model reported that H_2S production was increased in the heart muscle and this led to a profound protection against ischemia-reperfusion injury induced heart failure[32]. Finally, in 2014, King et al., characterized global CSE KO murine hearts in response to ischemia reperfusion injury. They found that CSE KO mice exhibit elevated oxidative stress, dysfunctional eNOS, diminished NO levels and exacerbated myocardial infarction injury[33].

As described from the later study, there is evidence for an interplay between the two gasotransmitters H_2S and NO in the context of ischemia-reperfusion injury. The cardioprotective effects of H_2S have been reported to be nitric oxide (NO)-dependent and this was claimed to occur in a cGMP-independent manner[33]. The possible interplay has already been described in other systems and pathologies (i.e.[40])

1.4.3. The role of NO in the pathophysiology of ischemia-reperfusion injury

Nitric oxide synthases (NOS) are the enzymes responsible for NO generation. To date, three distinct NOS isoforms have been identified: neuronal NOS (type 1), inducible NOS (type 2) and endothelial NOS (type 3). NOS's catalyze an overall 5- electron oxidation of one NN-atom of the guanidino group of L-arginine to form NO and L-citrulline, with the intermediate NN-hydroxy-L-arginine (NOHA). NO synthesis is critically influenced by various cofactors such as tetrahydrobiopterin (BH4), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)[41].

We have to mention here, that although the studies reported below on the involvement of endogenous NO in the pathophysiology of ischemia-reperfusion injury are numerous, the role of endogenous NO remains still controversial.

In addition, the mechanisms underlying NO protection are not yet clear. In brief, NO confers to cardiomyocyte survival due to its capability of inhibiting the opening of mitochondrial permeability transition pore (mPTP) and the cardiac mitochondrial voltage-dependent anion channel (VDAC)[42]; elevating cGMP-dependent signaling[43] or direct redox-related Ca^{2+} handling protein modification (S-nitros(yl)ation)[44]. However, the only evidence of how endogenous NO confers cardioprotection involves the translocation of PKC-alpha, -delta and -epsilon isoforms to the nucleus-myofibril fraction and the translocation of PKC-alpha to the

membrane fraction after ischemia (20 min) and reperfusion (10 min) in the perfused rat heart. The authors suggested that NO generated during reperfusion following ischemia activates PKC isoforms and may protect the heart against contractile dysfunction in the perfused rat heart[45].

1.4.3.1. NOS activity studies during ischemia-reperfusion injury.

According to the existing literature, changes in NOS activity during ischemia-reperfusion injury seem to be species specific and highly contradictory.

The first evidence of the involvement of endogenous NO, but only superficial came on 1992. Matheis et al., reported that in in vivo piglets a reduction on circulating NO in systemic venous and coronary sinus blood content during hypoxia which increased during reperfusion, indicating reduced NOS activity during ischemia[46]. Following this observation in in vivo pigs a 90% reduction in Ca^{2+} dependent NOS activity was observed in ischemia-reperfusion versus non ischemic hearts. It was also suggested that L-arginine administration increased specifically eNOS activity leading to a reduction of infarct size[47]. However, contradictory findings on eNOS activity during ischemia –reperfusion has been reported in the isolated rat model even from the same authors. In brief the group of Zweier[48] by performing EPR studies with a spin trap for NO, found that $NO^{\cdot}$ increased during ischemia; results replicated also by a different group[49], which reported that that tissue NO signal is increased after 30 minutes of ischemia. A little later the Zweier's group on 1996 suggested that in the 20th second of reperfusion the NO effluent of the isolated ischemic rat heart was reduced until the 5th minute of reperfusion[50]. Contradictory findings were further reported as it was suggested that in longer ischemic periods of 30 minutes eNOS activity is reduced and recovers after 45 minutes of reperfusion[51]. Finally the same group in the most recent study suggested that eNOS activity is reduced constantly during the ischemic period. As for the rabbit isolated heart it has been proved that cytosolic eNOS activity is increased during ischemia[52]. Activation is rapid, persists during the whole ischemic episode and disappears during reperfusion[53].

However, except the study on in vivo pigs, in vivo data on the NOS activity during myocardial infarction do not exist. Measurement of NOS activity in vivo is tricky, and usually studies assess the levels of the surrogate marker of eNOS activity, increased cGMP levels. In this context the only study supporting in vivo evidence of reduced eNOS activity during ischemia showed reduced cGMP myocardial concentration[54]. Although, the authors pointed that this cannot be explained.

1.4.3.2. Lessons from NOS inhibition studies and knockout models on the involvement of NO in ischemia-reperfusion injury

The results on this topic are extremely controversial. It is important before moving to the reported literature to differentiate i) the endogenous produced NO from the exogenous administrated and ii) the role of endogenous NO in the non-adapted (non-conditioned) myocardium.

The first assess of the role of endogenous NO on ischemia reperfusion injury was made due to enhancement of NOS activity by administration of L-arginine (the potent substrate of NOS) during the injury. Studies reveal a cardioprotective role of enhanced NOS activity as L-

arginine improves endothelial function and reduces myocardial infarction in in vivo pigs[55], rats[56]; cats[57] and dogs[58, 59].
The second assess was made by inhibiting NOS during myocardial infarction with the administration of NOS selective inhibitor L-NAME. Studies herein are controversial. Only one of them support that L-NAME increases myocardial infarct size in perfused rat hearts[45]. However, most of them done in isolated ischemia-reperfusion models support a cardioprotective role of L-NAME, as NOS inhibition reduced infarct size in rats[60] and rabbits[61-63].

These controversial findings on NOS and NO involvement in the pathophysiology of ischemia- reperfusion injury are also obvious in knockout models. Interestingly, in 1999 Jones et al., reported that eNOS endothelial specific KO have increased infarct size[64], a finding contradictory to a most recent study[65]. In the later study, two distinct murine models of transgenic overexpression, a cardiomyocyte-specific eNOS overexpresser (CS eNOS-Tg) under the control of the alpha-myosin heavy chain promoter, and a systemic eNOS transgenic mouse (SYS eNOS-Tg) under control of the native eNOS promoter with an upstream endothelial enhancer element., were developed. The authors induced 30 or 45 minutes of LAD ligation. They found that CS eNOS-Tg mice displayed significantly decreased infarct size beyond that of mice with systemic overexpression, indicating that the endothelium derived NO has no effect on myocardial infarct size compared to the cardiomyocyte derived NO[65]. These are in line with other observations supporting that murine hearts overexpressing eNOS may be maximally protected against ischemia-reperfusion injury by their elevated endogenous NO levels[66]. In addition to this, application of myocardial ischemia-reperfusion injury on global eNOS knockout models made things more complex. Sumeray et al., reported that eNOS KO suffered larger infarcts[67], whilst other groups [68,56] suggested that eNOS KO mice had smaller infarct size than its wild type littermates.

1.4.3.3. eNOS specific post-translation regulation and functional effects on the pathophysiology of ischemia-reperfusion injury

Endothelial nitric oxide synthase (eNOS) is constitutively expressed as already described, however functionally numerous post-translational modifications regulate its activity in vitro and in vivo[69, 70]. Over the last 10 years, it has become clear that phosphorylation of eNOS on serine, threonine and tyrosine residues determine the enzyme's capability to produce NO[71]. Among the numerous putative phosphorylation sites most is known about the activatory role of Ser1177 (human sequence), which is situated in the reductase domain of the enzyme and the inhibitory role of Thr495, located in the calmodulin (CaM)- binding domain, the phosphorylation of which appear to play a reciprocal role in the regulation of NO production[72, 73].

It is also, clear that the enzyme can be tyrosine-phosphorylated in endothelial cells treated with tyrosine phosphatase inhibitors[74, 75]; H_2O_2 [75, 76], or exposed to fluid shear stress[77] as well as in cells overexpressing v-Src[75, 78] on Tyr81. eNOS Tyr81 phosphorylation increases eNOS activity[76, 79]. Recently a novel mechanism of tyrosine eNOS phosphorylation has been described. It was reported that Tyr657 in the FMN domain of eNOS is a critical determinant of its enzymatic activity. In brief, the authors suggested that the phosphorylation on Tyr657 by proline-rich tyrosine kinase 2 (PYK 2) under fluid shear stress decreases eNOS activity, and the mutation of Tyr657 to a phosphomimetic glutamate or aspartate residue completely abolished NO production[80].

Activation of the phosphoinositide-dependent kinase 3 kinase (PI-3K)/Akt axis leads to Ser1177 eNOS phosphorylation and enhances NO production; this pathway constitutes a major protective pathway in ischemia-reperfusion injury in the heart [81]. The beneficial effects of eNOS-derived NO have been demonstrated both in isolated perfused heart preparations and *in vivo* by pharmacological, genetic and gene-therapy approaches[82-84]. Endogenously generated and exogenously applied NO exerts its beneficial effects either through cGMP/PKG-dependent pathways or by directly targeting the mitochondria[85, 86]. The former pathways have been shown to involve among others K_{ATP} channels, the sodium–proton exchanger and PKCε leading to inhibition of mitochondria permeability transition pore opening[81, 87, 88], while the latter relies on S-nitrosation of complex I to reduce ROS production[89].

Although the regulation of eNOS by phosphorylation is widely studied in the endothelium limited knowledge exist on the regulation of the synthase in heart and especially in the pathophysiology of ischemia reperfusion injury. The majority of the studies report that eNOS phosphorylation on Ser1177 is the indicator of increased eNOS activity conferring to cardioprotection. However, a limited number of studies evaluate eNOS regulation during ischemia or/ and reperfusion injury in the heart. In 2011 Cai et al reported that in the in vivo model of murine ischemia reperfusion injury, eNOS phosphorylation on ser1176 was decreasing in a time dependent ischemic manner; 30 minutes of LAD ligation reduced eNOS ser1176 phosphorylation, 45 minutes reduced it even more with maximal reduction in 60 minutes of ischemia (irreversible damage). The authors linked the time dependent decrease on eNOS phosphorylation-named by them as eNOS activity (although they did not measure directly eNOS activity) to increased infarct size caused by ischemia[90]. In 2013 Inserte et al[91]., reported that in a isolated rat model of ischemia reperfusion injury eNOS at ser1176 was phosphorylated under basal conditions. 40 minutes ischemia reduced this phosphorylation. Reperfusion, in which ROS are acutely formed and released[51] resulted in a time dependent increase of ser1176 phosphorylation[91]. However these findings are contradictory to a former study by Chen et al. who suggested that in short ischemic insults (10 minutes) eNOS Ser phosphorylation and activity has been shown to be increased in a AMPK dependent manner[92].

PYK 2 (proline-rich tyrosine kinase), also known as RAFTK, CAK, and CADTK is the cytoplasmic tyrosine non receptor kinase and exhibits 45% amino acid sequence identity to focal adhesion kinase. Tyrosine phosphorylation of PYK 2 and focal adhesion kinase was triggered by integrin-mediated adhesion. PYK 2 was stimulated by a broad range of physiological stimuli such as stimuli for G-protein– coupled receptors that elevate intracellular Ca^{2+}, inflammatory cytokines, and stress signals[93].

In the ischemic and reperfused heart, there is a marked increase in oxygen radical generation[94, 95]. Radical generation occurs within both the endothelium and myocytes[96, 97]. EPR studies have provided direct detection of free radicals in the ischemic and reperfused heart and demonstrated that $\bullet O_2^-$ and hydroxyl radicals are formed[94]. So, a better understanding on the effects of ROS-PYK2 pathway on eNOS regulation in myocardial infarction is necessary. Additionally, the role of eNOS phosphorylation on Tyr657 mediated by PYK2 has never been studied in vivo.

Finally, two studies suggest that transgenic mice[98] or pigs in gene therapy expressing a phosphomimetic S1176D form of eNOS showed improved myocardial reperfusion and significantly reduced infarct size[99].

However, we have to keep in mind that the role of eNOS regulation by multiple kinases in the context of myocardial ischemia reperfusion injury is completely not understood. From the foregoing discussion, it is clear that total injury sustained by a tissue represents the sum of damage attributable to ischemia *per se* plus that invoked by reperfusion. Importantly, it is clear that the reperfusion phase is very dynamic and that cell death can continue for up to 3 days after the onset of reperfusion[100]. Thus, understanding the mechanisms involved paves the way for development of novel therapeutic opportunities that not only reduce the extent of injury induced by ischemia reperfusion but may also extend the time which a tissue could be subjected to ischemia before irreversible injury occurs. The latter point has important implications for organ transplantation, cardiopulmonary bypass, and operation in a bloodless field.

General Introduction

Part II: Cardioprotective Strategies.

As discussed in detail in Part I, ischemia–reperfusion injury is the most decisive factor in myocardial impairment in patients. Developing new strategies to reduce ischemia–reperfusion injury (Figure 5) is currently one of the main goals of research in cardioprotection. In this context, myocardial ischemic conditioning has been proposed as an interesting adjuvant to classical cardioprotection during myocardial infarction. Myocardial ischemic conditioning is a powerful endogenous cardioprotective phenomenon. It was first described in animals in 1986. During the last thirty years, it has been the subject of considerable research concerning both its underlying mechanisms and its clinical applications. Several clinical trials in cardiac surgery and interventional cardiology have therefore sought to demonstrate its effectiveness in clinical settings[101].

1.1. Conditioning Phenomena

After the pioneering observations that reperfusion indeed salvages ischemic myocardium from infarction[102, 103] and that infarction spreads in a wavefront during ongoing ischemia but leaves salvageable myocardium up to 2 to 3 hours of ischemia[104, 105], the prime cardioprotective paradigm of ischemic preconditioning (PreC) was established by Murry et al[106], who reported that 4 cycles of 5 minutes coronary occlusion/5 minutes reperfusion immediately before a sustained 40 minutes coronary occlusion with reperfusion reduced infarct size; however, infarct size was not reduced when the sustained coronary occlusion was of 3 hours duration, emphasizing the need for timely reperfusion[107]. The observed infarct size reduction was greater than anything that could be achieved by even combined pharmacological treatment. Initially, this cardioprotective phenotype was attributed to metabolic adjustments, notably slowed energy metabolism[108]. It was the landmark study by Downey and collaborators[109] who identified the action of adenosine as a signaling event in such PreC and opened the avenue for all subsequent studies on cardioprotective signaling. PreC comes in 2 different temporal forms, that is, an acute form which confers immediate protection but disappears after an interval of ≥2 hours between the preconditioning stimulus and the event which needs protection[106] and a delayed form which reappears after 24 to 48 hours, lasts longer but is less protective in strength[110, 111].

The acute form of PreC relies on the recruitment of acutely available signaling modules, whereas the delayed form involves increased expression of protective proteins in response to an acute signal[112]. PreC has been successfully translated to humans with ischemic heart disease, but because of its nature (“pre”) can only be used in elective settings, such as percutaneous coronary interventions and coronary artery bypass grafting and not in acute myocardial infarction[113]. Ischemic postconditioning (PostC) was established by the group of Vinten-Johansen[114] who first reported that 3 cycles of 30 seconds reperfusion/30 seconds re-occlusion at the immediate onset of reperfusion after 60 minutes coronary occlusion reduced infarct size to an equivalent amount as PreC. This seminal study once and forever terminated the long-lasting and controversial debate whether reperfusion injury contributed to ultimate infarct size[115]; moreover, this study unequivocally demonstrated that reduction of infarct size with modified reperfusion was possible[116] and thus revitalized prior debate on gentle reperfusion. PostC is largely limited to the early minutes of reperfusion. PostC has been successfully translated to humans with ischemic heart disease, and as an intervention that is performed at immediate reperfusion, it can be used in patients undergoing interventional reperfusion of acute myocardial infarction[113]. Therefore, a better understanding of the signal transduction underlying the conditioning phenomena may help to recruit cardioprotection

without the inevitable injury associated with ischemia/reperfusion and also without manipulation of the culprit coronary lesion. Understanding the signal transduction may also help to account for and attenuate interference from confounding risk factors, comorbidities, and comedications[117].

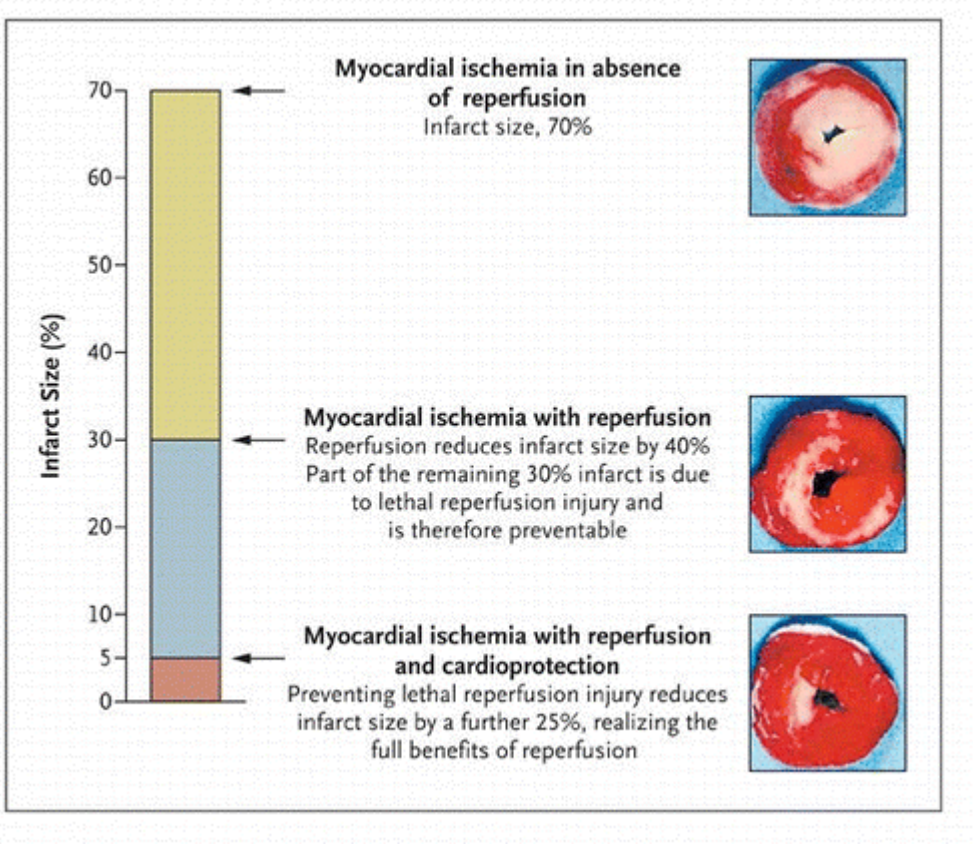


Figure 5: Illustration of the concept of reperfusion injury. During myocardial ischemia, cell death progresses. Reperfusion halts this process, but its benefit is limited by an increase in cell death during the first few minutes of reperfusion; this death can be avoided by using cardioprotective treatments at the time of flow restoration[17].

1.2. Signaling Molecules and Mechanisms in Conditioning

There are now thousands of studies which reported >100 different signaling molecules and mechanisms of conditioning in a wide range of experimental preparations[101]. The ultimate end point of protection is reduction of infarct size.

1.2.1. Stimuli/Triggers of Conditioning

1.2.1.1. Chemical Stimuli

Endogenously released chemical stimuli which elicit cardioprotection include small molecules, such as calcium ions, reactive oxygen species (ROS), reactive nitrogen species

(RNS), hydrogen sulfide, and more classical ligands which activate sarcolemmal receptors. Extracellular calcium ions can precondition through adenosine and protein kinase C (PKC) activation[118, 119], but their role in ischemic conditioning phenomena is probably minor. ROS have an ambivalent role in the conditioning phenomena: whereas excess formation of ROS contributes to irreversible injury, small amounts of ROS, for example, in response to mitochondrial K_{ATP} activation or mPTP opening contribute to protection, possibly through oxidation of protective cytosolic kinases[120]. ROS share the same paradox with the conditioning phenomena per se, in that a little ischemia/reperfusion or ROS protects, whereas more profound ischemia/reperfusion or ROS formation induces injury. RNS, notably NO, also share this dose-dependent paradox[41]. Small concentrations of NO improve ventricular function[121] and the matching of oxygen consumption to contractile function[122] whereas high concentrations of NO depress contractile function[123].

1.2.1.2. Autacoids

Autacoids, such as adenosine and bradykinin, are released from cardiomyocytes, endothelium, and interstitial cells during the preconditioning ischemia/reperfusion cycle(s)[124]. Interstitial adenosine concentrations are increased and enzymatic catabolism by adenosine deaminase attenuates cardioprotection[125]. Adenosine activates PKC directly[126] and it activates the downstream reperfusion injury salvage kinase (RISK) and endothelial NOS/protein kinase G (PKG) pathways[127]. Cardiomyocytes express adenosine A_1, A_2A, A_2B, and A_3 receptors on their sarcolemma; for the A_2B receptor, there seems to be also a mitochondrial localization [128]. A_1 and A_3 receptors are essential for PreC, and their blockade abrogates protection [129]. In contrast, A_2A and A_2B receptors are essential for PostC, and they must be activated during the early minutes of reperfusion to achieve protection[130]. However, results on the involvement of adenosine receptors in cardioprotection are controversial. In our hands non selective A_1 and A_2B but not A_2A and A_3 ARs agonists are essential for triggering cardioprotection, activating RISK and survivor activating factor enhancement (SAFE) pathway[131]. There seem to be species differences as the reduction of infarct size by exogenous adenosine is still controversial, among rodents and larger mammals[132].

Bradykinin is cleaved from kininogen precursors in the interstitium and catabolized through the angiotensin-converting enzyme in the vasculature but also through neutral endopeptidase in the interstitium[133]. During preconditioning ischemia-reperfusion cycle(s), the interstitial bradykinin concentration is rapidly increased[124]. Bradykinin receptor 2 blockade abrogates protection by PreC and PostC[134].

1.2.1.3. Neurohormones

Neurotransmitters and hormones, such as acetylcholine catecholamines, endothelin and opioids[135], can induce cardioprotection after exogenous administration and activation of their respective receptors[136]. However, only α-adrenoceptor activation is causally involved in the conditioning phenomena through formation of adenosine and activation of PKC in PreC, whereas its blockade abrogates protection[137]. Opioids are released acutely from nerve endings but also synthetized in cardiomyocytes. The δ receptor is most important in the conditioning phenomena, that is, not only nonspecific opioid receptor blockade with naloxone abrogates protection by PreC[138], but also specific δ receptor blockade largely abrogates protection by PreC[138] and PostC[139].

1.2.1.4. Lipid Molecules

Several lipid molecules which activate G protein-coupled receptors have been implicated in the conditioning phenomena; however, their role as triggers or mediators is not really clear. Prostaglandins are causally involved in PreC and PostC, and cyclooxygenase (COX) inhibition abrogates protection[140]. Selective epoxyeicosanotrienoic acid antagonists abrogate the protection by PreC and PostC[141]. Sphingosine- 1-phosphate (S1P) is produced from the sphingosine kinase 1 isoform during PreC[142] and PostC[143] and reduces infarct size by activation of S1P1 and S1P3 receptors and subsequent activation of the RISK pathway[143]. The sphingosine kinase isoform 2 is also important for cardioprotection by PreC, as their genetic deficiency abrogates protection[144] possibly secondary to increased susceptibility to mPTP opening[144].

1.2.1.5. Cytokines/Chemokines

In contrast to peptide hormones and growth factors, cytokines/ chemokines have a causal role in PreC and PostC. The prototypic cytokine tumor necrosis factor a (TNFα) reduces not only infarct size following exogenous administration[145] but is mandatory for PreC[146] and PostC[147]. The protection is mediated through activation of TNF receptor 2, STAT 3, and mitochondrial K_{ATP}-channels[145].

1.3. Intracellular Mediators of Conditioning

1.3.1. Protein Kinase C

Abrogation of protection by PreC with 3 different PKC inhibitors it has been reported[148]. PKC is phosphorylated, activated, and translocated during PreC[148]. Subsequently it was reported that (1) there is interaction of PKC with other cytosolic kinases, notably protein tyrosine kinases[149-151], (2) different isoforms of PKC serve contrasting functions[152], and (3) intracellular translocation/colocalization of different isoforms is important[152].

In rodents, the PKCε isoform is most important for protection by PreC[153], and PKCε is translocated to the mitochondria through a heat shock protein–dependent import[154], where it activates mitochondrial K_{ATP} channels during PreC[155]. PKC activation, notably of its ε isoform, is also causally involved in PostC, where it again interacts with mitochondrial K_{ATP} channels and ROS formation[156]. Of note, there is a positive feedback loop between PKC activation and the trigger signal adenosine, in that PKC sensitizes the A_2B receptor during early reperfusion and thereby contributes to PreC[157].

1.3.2. NO and Protein Kinase G

Endothelial nitric oxide synthase (eNOS) is activated after G protein–coupled receptor activation through a sequence of signals involving PI3K, phosphoinositide-dependent kinase, and protein kinase B (Akt)[158-160] which then phosphorylates and activates eNOS[161]. The resulting NO activates soluble guanylate cyclase to form cGMP, which then activates PKG. NO signals protection by PreC not only through PKG, but also through nitrosylation[162, 163] and the PKG-independent action of NO seem to occur at reperfusion[164].

PKG is causally involved in PreC[87] and PostC[165]. Several studies have suggested a beneficial role of cGMP/PKG-regulated pathways in PostC[87, 91, 166, 167]. PKG activation exerts anti-apoptotic effects through PKCε activation and inhibition of mPTP opening[87]. In addition, increased cGMP levels and PKG activation lead to attenuated contractility during reperfusion through altered calcium handling[87, 166, 168]. In two recent studies, it was shown that the beneficial effects of PKG result from i) delayed normalization of intracellular pH during reperfusion through altered NHE function[167] and ii) antioxidant effects that prevent eNOS uncoupling[91]. Two main targets of PKG activation are K_{ATP} channels with subsequent ROS formation (included in the effectors section[165]) and Ca^{2+} handling proteins[165], that have been correlated to conditioning phenomena. Among the Ca^{2+} handling proteins phospholamban (PLN) is a downstream substrate of PKG[87, 169, 170]. Moreover, a number of publications have shown altered PLN phosphorylation in the context of ischemia-reperfusion[168, 171-173]. In a recent study Inserte et al., showed that phosphorylation of PLN (ser16/thr17) peaked in the control group 3 min after the onset of reperfusion and declined thereafter. On the other hand, following PostC increased PLN phosphorylation was evident in the 5th min of reperfusion. It was, thus, proposed that delayed PLN phosphorylation limits reperfusion-triggered Ca^{2+} oscillations leading to protection[168]. PLN once phosphorylated by PKG reduces free intracellular Ca^{2+} concentration by dissociating from sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA); SERCA is then able to pump Ca^{2+} ions back into the SR[174].

1.3.3. RISK Pathway

Activation of PI3K and its downstream targets Akt and glycogen synthase kinase 3β (GSK 3β) by PreC[175, 176] and activation of extracellular signal-regulated kinases (ERK) by the PreC trigger adenosine, and subsequent transactivation of the epidermal growth factor receptor[177] was demonstrated after the preconditioning cycles and found mandatory for infarct size reduction. Importantly, elegant studies by Hausenloy and Yellon then reconciled the studies on the role of RISK activation by PreC and by exogenous agonists during reperfusion; they demonstrated a biphasic RISK activation response during the preconditioning cycles and again at early reperfusion[178]. The importance of the RISK pathway was further supported by studies demonstrating a causal role of PI3K, Akt, and further downstream kinases, such as p70 S6 kinase and GSK 3β in PreC[179] and in PostC[180, 181], including studies in isolated human right atrial trabeculae[182]. GSK 3β was proposed to be the downstream point of convergence of the RISK pathway, which when phosphorylated and thus inhibited acts to inhibit mPTP opening[179, 183]. Recent studies also demonstrated morphological protection of mitochondria by Akt activation[184]. Collectively, from these studies, the intriguing concept emerged that RISK activation at early reperfusion is a unifying pattern of cardioprotective signaling. Some caveats remain: Genetic ablation of GSK 3β did not abrogate protection by PreC or PostC in one study[185], but GSK 3β was mandatory for PostC in another study using the same animal model[186], such that the pivotal role of GSK 3β remains controversial. Also, although the RISK pathway as such is obviously mandatory for protection in rodents, it is not for PostC in pigs[187].

1.3.4. SAFE Pathway

Infarct size reduction by PreC and also in response to exogenous TNFα was abrogated in STAT 3–deficient mouse hearts[188], and STAT 3 activation—in interaction with Akt activation[189]—was mandatory for PreC. The coronary effluent from a preconditioned heart also activates STAT 3 in the acceptor heart and induces functional protection[190]. PostC also relies on STAT 3 signaling in mice[191] and pigs[192]. Protection by PostC also involves TNFα and its receptor 2 activation upstream of STAT 3[147]. Collectively, TNFα, its receptor subtype 2, and STAT 3 were then viewed as the SAFE system[193]. STAT 3 activation not only has a role in more long-term transcriptional upregulation of cardioprotective proteins[194], but also an acute effect to improve mitochondrial respiration[195], notably during PostC[192] to attenuate apoptosis.

1.3.5. MicroRNAs

MicroRNAs are important signaling molecules not only in cardiovascular development and differentiation, but also in myocardial ischemia-reperfusion injury[196]. Several different microRNAs have been identified in protocols of PreC[197-200] and PostC [200, 201] in various species, including humans[202, 203]. The emerging field of microRNAs in cardioprotective signaling is promising, but requires further robust data with cause- and -effect relationships.

1.4. Effectors of Cardioprotective Conditioning

1.4.1. Mitochondria

There is unequivocal consensus that mitochondria are the most important effectors of conditioning's protection, where most, if not all, of the above signaling pathways converge. The mitochondria are decisive for cellular survival or death. They provide the ATP for the maintenance for ionic gradients and, thus excitability and excitation-contraction coupling, for the contractile machinery, and for the maintenance of cellular integrity. Ischemia with its inherent lack of supply of oxygen as an electron acceptor inhibits the flow of electrons along the respiratory chain, induces inner mitochondrial membrane depolarization, and limits the formation of ATP. Opening of the mPTP on reperfusion initiates a deleterious chain of events. The mitochondria are, however, equipped with several modules, which inhibit mPTP opening[204].

The mPTP is a large-conductance megachannel in the inner mitochondrial membrane which—when open for longer terms— dissipates the inner mitochondrial membrane potential, results in matrix swelling, rupture of the outer mitochondrial membrane, and release of cytochrome C from the intermembrane space into the cytosol where it activates proteolytic processes and initiates cellular disintegration[204]. The molecular identity of the mPTP is still unclear. Although mPTP was traditionally thought of as a multimeric complex of voltage-dependent anion channels of the outer membrane in interaction with adenine nucleotide translocase of the inner membrane, which was regulated by cyclophilin D in the matrix[205], all of these individual constituents seem to be dispensable[206] and the mPTP is more recently considered to be formed from F-ATPase[207]. Inner membrane depolarization, high concentrations of inorganic phosphate, ROS, and RNS are all present during myocardial ischemia and more importantly during reperfusion[208] therefore favor mPTP opening. Transient mPTP opening may serve a physiological function in ROS homeostasis[209] and

indeed transient mPTP opening is cardioprotective during PreC and PostC[210]. Thus, the genuine paradox of conditioning—a little injury protects, whereas profound injury is deleterious—characterizes also mPTP opening. No matter, what exactly the molecular composition of mPTP is, cyclophilin D regulates mPTP opening, and it decreases the threshold for mPTP opening in response to calcium and inorganic phosphate[204]. Cyclosporine A is a drug which inhibits cyclophilin D, thus inhibiting mPTP opening, and ultimately also reduce infarct size in experimental studies[211] but also in humans with reperfused acute myocardial infarction[212]. The mPTP not only plays a central role in mitochondrial and cellular death or survival, it is also a point of convergence of cardioprotective signaling. In particular, inhibition of GSK 3β was proposed to integrate all upstream signals and exert an inhibitory effect on mPTP opening[179].

1.4.2. K_{ATP} Channels

The K_{ATP} channel was first identified as a cardioprotective drug target[213] and only subsequently as a signaling element of conditioning[214]. Initially, the focus was on the K_{ATP} channel in the sarcolemma, which contributes to action potential duration shortening during myocardial ischemia and was therefore thought to alleviate cytosolic calcium overload[215]. However, the predominant site of cardioprotective signaling by K_{ATP} channels are the mitochondria, as first identified by Marban and collaborators[216]. The mitochondrial K_{ATP} channel is a target of NO[217], PKC[218] and PKG[219] and on its activation releases ROS which, in turn, activate the PKCε in a positive feedback loop[220]. It is not really known how the mitochondrial K_{ATP} channel–induced ROS release induces protection, other than further activating PKCε. PostC's cardioprotection depends on a signal transduction of PKC activation, mitochondrial K_{ATP} activation, and ROS release[165] very much alike PreC.

1.4.3. Protein Nitrosation/Nitrosylation

NO is an essential molecule in PreC and PostC, as detailed earlier. Not all of NO's actions in cardioprotection are mediated through increased cGMP formation and consequent PKG activation[164]. In fact, NO directly targets several proteins and alters their structure and function through nitrosation or nitrosylation[162] . The nitrosylation is thought to shield cysteine residues from oxidation[221]. PreC[163] and PostC[222] reduce infarct size independently from PKG, and several nitrosylated proteins were identified in preconditioned myocardium, notably also mitochondrial proteins[221].

1.4.4. Acidosis During Reperfusion

Acidosis during early reperfusion inhibits mPTP opening but also the development of hypercontracture[223]. Acidosis at early reperfusion is mandatory for PreC's reduction of infarct size, and protection is abrogated by sodium bicarbonate infusion[224]. To maintain acidosis at early reperfusion, the sodium–proton exchanger is inhibited by PreC[225]. Likewise, protection by PostC requires maintenance of acidosis at early reperfusion[226], and vice versa acidic reperfusion reduces infarct size[227]. Activation of PKG contributes to delayed recovery of acidosis during reperfusion through inhibition of the sodium–proton exchanger end results in the cardioprotection observed by PostC[228].

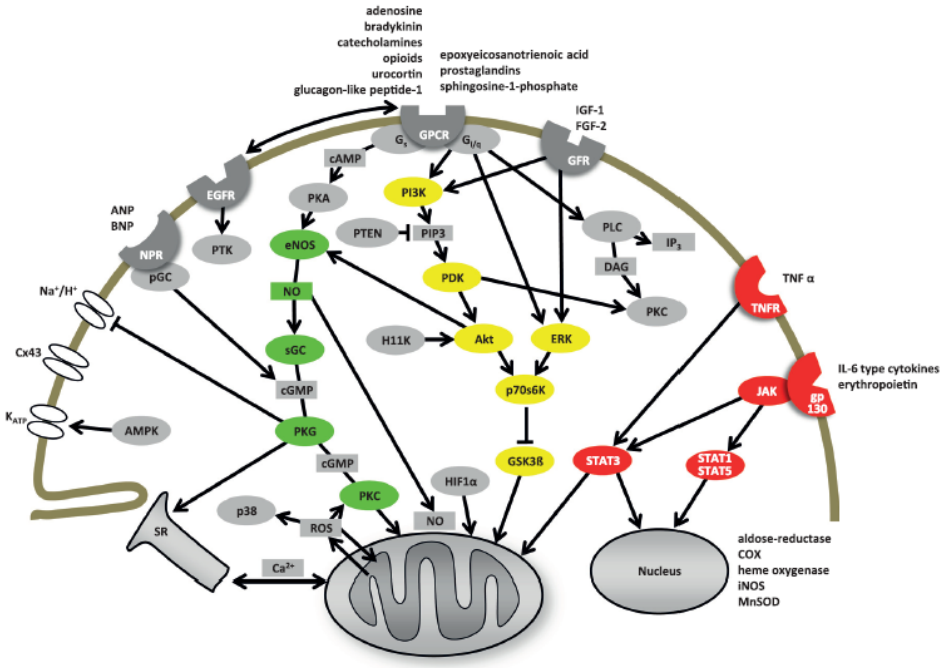


Figure 6: Simplified scheme of cardioprotective signal transduction. Akt indicates protein kinase B; AMPK, cyclic adenosine monophosphate–activated kinase; BNP, brain natriuretic peptide; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; Cx 43, connexin 43; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; FGF, fibroblast growth factor; Gs/Gi/q, stimulatory/inhibitory G protein; GPCR, G protein-coupled receptor; gp130, glycoprotein 130; GSK3β, glycogen synthase kinase 3 β; H2S, hydrogen sulfide; H11K, H11 kinase; HIF1α, hypoxiainducible factor 1α; IGF, insulin-like growth factor; iNOS, inducible NO synthase; IP3, inositoltrisphosphate; JAK, Janus kinase; KATP, ATP-dependent potassium channel; Na+/H+, sodium/proton-exchanger; NPR, natriuretic peptide receptor; pGC, particulate guanylate cyclase; p38, mitogen-activated protein kinase p38; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PI3K, phosphatidylinositol (4,5)-bisphosphate 3-kinase; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; PTEN, phosphatase and tensin homolog; PTK, protein tyrosin kinase; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SR, sarcoplasmic reticulum; STAT, signal transducer and activator of transcription; and TNFα, tumor necrosis factor α. The NO/PKG pathway is displayed in green, the RISK pathway in yellow, and the SAFE pathway in red[101].

1.5. Results from human studies on post-conditioning mechanism

Since 2005, several small, phase II trials in patients with ST-segment elevation myocardial infarction (STEMI) have demonstrated that ischemic postconditioning (during angioplasty) can reduce infarct size, attenuate myocardial oedema and microvascular obstruction during the early hours after reflow, and improve the recovery of contractile function several months after the initial insult[229]. These multicentre studies, performed in various countries, confirmed the large body of experimental evidence indicating that lethal reperfusion injury is of great importance (accounting for 30–40% of the final infarct size) and can be alleviated by a timely protective intervention[17]. In a review published in January 2013, Heusch reported that

ischemic postconditioning had been shown to be beneficial in 10 trials, two studies had neutral findings, and none of the studies reviewed showed a significant harmful effect of this therapy[113]. The POST trial[230] is the latest postconditioning clinical trial performed, and has the largest sample of patients with acute STEMI to date. This trial fell short of showing any significant benefit from postconditioning. Data from large-scale studies have suggested that conditioning interventions might improve clinical outcomes[231]. However, as recently reported, the results of the CIRCUS (Does Cyclosporine Improve Clinical Outcome in ST-Elevation Myocardial Infarction Patients) trial showed no significant protection by CsA in the primary clinical outcome. Pharmacological reasons linked to CsA for the results of the clinical trial include pharmacokinetics and pharmacodynamics of the drug. For example, a limited diffusion of the drug in the area at risk, a low tissue diffusion of the compound, hydrophobic characteristics, blood cell capture and high metabolism could be suggested as reasons for the negative results. The results of the CIRCUS trial could contribute to the withdrawal of the mPTP blockade pharmacological strategy as a way to protect the myocardium from ischemia-reperfusion injury. Nevertheless, if many pharmacological aspects insights could have taken under consideration, this would have contributed to positive results of the clinical trial[232].

Considering the overall low rate of ST-segment resolution and the questionable technique of angioplasty post- conditioning in the POST trial[230] one might wonder whether the absence of protective effect was, at least in part, a result of inadvertent microembolization during repeated balloon inflations and deflations. At most, the data from these trials reinforce the need for a study driven by hard clinical outcomes, and re-emphasizes the need for carefully conducted reperfusion procedures in the catheterization laboratory

1.6. Pharmacological Conditioning

Our increasing understanding of the mechanisms underlying ischemic conditioning has identified a vast array of signaling mediators, which can be targeted by pharmacologic agents to recapitulate the cardioprotective effects of ischemic conditioning. In this regard, a number of pharmacologic approaches to limit infarct size in STEMI patients undergoing primary PCI have been investigated[233]. Unfortunately, many of these studies have failed to demonstrate any cardioprotective effect in the clinical setting, despite promising experimental animal data. This apparent failure can be attributed to a number of different factors. These include the use of animal models that do not adequately represent clinical reality, e.g., due to lack of comorbidities, and poor study design[233-235].

More recently, several novel pharmacologic approaches have been reported to limit infarct size when administered before reperfusion in primary PCI-treated STEMI patients[117]. Most promising among these therapies are cyclosporine-A, exenatide, and metoprolol. Whether these pharmacologic postconditioning agents can actually improve clinical outcomes remains to be investigated, as the most recent results from the huge multicenter study CIRCUS failed to show protective effects in humans.

In summary, mechanical and pharmacologic conditioning strategies are promising therapeutic options for cardioprotection in patients undergoing elective or emergency coronary revascularization, although there are several negative studies. Most of the clinical trials, both positive and negative, use limited number of patients. The positive trials have been conducted in selected patients under well controlled conditions, whereas the negative trials (e.g., on remote preconditioning) have been less selective in terms of patient recruitment and

procedures (anesthesia, surgery). The observed lack of protection in the negative studies can in part be attributed to the presence of different risk factors, comorbidities, and their medications in different patient cohorts, as well as to poorly validated drug targets in juvenile and healthy animal models, and poorly designed clinical studies[236]. None of the existing studies has really raised a safety concern for the conditioning strategies. Larger studies with clinical outcome endpoints are necessary to gain more insight into the clinical applicability of conditioning strategies in different patient populations with different medications and confounding factors.

1.6.1. Pharmacological postconditioning afforded by gasotransmitters

1.6.1.1. Role of exogenous administrated NO in mimicking post-conditioning mechanisms

Since NO is a cytoprotective molecule, NO donor drugs including NO gas itself are promising tools for pharmacological cardioprotection.

NO gas inhalation during coronary occlusion has been shown to provide infarct size reduction and leads to a decrease in peroxynitrite formation in rats and mice[237] showing that NO inhalation may represent a promising PostC mimetic in acute myocardial infarction patients.

Organic nitrates, as NO releasing drugs are promising tools in the context of cardioprotection. Among them glyceryl-trinitrate (commonly termed nitroglycerin-NTG); is a potent nitro-vasodilator, which has been shown to increase NO species under clinical conditions[238]; it has been used for the prevention and treatment of ischemic heart disease for more than 100 years. The positive effects of NTG arise from its ability to promote vasodilation, resulting in increased blood flow to the heart. NTG's effects are also evident in systemic veins where the venodilator effect reduces cardiac preload and further decreases myocardial wall stress[239]. NTG is extremely effective in restoring the equilibrium of oxygen and nutrients supply-demand in the ischemic heart. However, sustained NTG administration causes tolerance and is associated with pro-oxidant effects, endothelial dysfunction and increased sensitivity to vasoconstrictors[240, 241]; furthermore it causes tolerance to ischemic preconditioning effects[242, 243]. Although, it has been shown that the protective effect of PreC can be mimicked pharmacologically with nitric oxide (NO) donors[244]; the effect of NO donors as pharmacological postconditioning agents has only superficially been assessed.

In brief, exogenous NO donors (SNAP) are cardioprotective when given during ischemia in isolated rabbit hearts[164]. In contrast IV administration of nitroglycerin has not been proved to be cardioprotective when it is administered during ischemia and reperfusion in anesthetized pigs[245] and in anesthetized rabbits[246]. In the former study of Liu et al., the mean arterial pressure decreased during the early reperfusion period in the NTG group and was even more decreased until the end of the reperfusion period, suggesting a progressive decline of LV function upon NTG administration in their model. However, in a in vivo canine model of ischemia reperfusion administration of nitroglycerin during the ischemic insult resulted in a reduction of myocardial infarction without affecting the haemodynamic profile of the studied animals[247].

Finally, several NO-modulating known drugs have been developed and investigated for cardioprotection. More interestingly, however, pravastatin, in contrast to the same dose of simvastatin or ischemic postconditioning, reduces infarct size in hypercholesterolemic rabbits

independently of its lipid lowering action, potentially through eNOS activation and nitro-oxidative stress attenuation[248].

However, although some functional studies on the effects of NO donors on ischemia – reperfusion injury have been published, there is completely lack of mechanisms underlying this protection. In conclusion, evaluation of the mechanisms underlying organic nitrates (that are already used in clinical routine) in the context of ischemic post-conditioning is mandatory.

1.6.1.2. Role of exogenous administrated H_2S in mimicking post-conditioning mechanisms

In vitro findings suggest a PostC mimicking effect of H_2S releasing sodium salts, when administrated on cultured cardiomyocytes during the reoxygenation period. For the first time, H_2S beneficial effects were linked to mitoK_{ATP} channels activation during reoxygenation[249]. These findings were further replicated in isolated heart models. In these studies the mechanisms underlying H_2S induced cardioprotection were evaluated; mitoK_{ATP} channels, Akt and PKC activation and the possible interplay among H_2S and eNOS in conferring infarct limiting effects were reported [249-251].

In the limited number of *in vivo* studies, administration of H_2S releasing agents during sustained ischemia has been shown to be beneficial in infarct size limitation and in post-infarction myocardial function[33, 36-38]. This cytoprotection was associated with up-regulation of antioxidant pathways[36], inhibition of myocardial inflammation[37], preservation of mitochondrial structure and function[37] after ischemia and inhibition of apoptosis[37, 38]. Whilst the cardioprotective effects of H_2S have been reported to be nitric oxide (NO)-dependent, this was claimed to occur in a cGMP-independent manner[33]. In the studies where H_2S was administered at a clinically relevant time (at the end of ischemia and during reperfusion)[33, 36-38], the intracellular signalling pathways determining H_2S-induced cardioprotection were not studied in detail. The positive preclinical findings with H_2S donors, have served as the impetus for a clinical trial designed to evaluate the cardioprotective potential of H_2S in humans (NCT01989208).

However, as described H_2S field is in its infancy and further evaluation of the signal transduction upon H_2S-releasing drugs administration in the ischemic heart are mandatory.

General Introduction

Part III: Comorbidities in cardioprotection.

Conditioning techniques have not become part of routine clinical practice, potentially for two reasons. First, many of the trials performed over the last two decades were small, single-center studies, frequently with few participants and a short follow-up period. As such, they have lacked the power needed to investigate the hard end points of death or a major cardiovascular event. Second, the effectiveness of ischemic-conditioning strategies in humans seems to be less profound than reported in the animal literature, with some randomized clinical trials showing no significant benefit[252, 253].

A possible explanation for this is the effect of underlying comorbidities on the ability of tissues to respond to the beneficial effects of ischemic conditioning. When extrapolating from animal models to humans it is vital to understand the differences between animal models and patients. Animals used for in vivo work are often juvenile, derived from inbred strains, of the same age and health, housed in the same environment with identical diets, and have no comorbidities.

Compare this to individuals with cardiovascular disease who participate in clinical trials, who are typically older, with comorbidities including diabetes, hypertension and kidney disease, and are taking several medications. Could it be that this disparity is the key to understanding why ischemic conditioning strategies fail to translate from animals to humans?

3.1. Hypercholesterolaimia

Hypercholesterolemia was the first comorbidity reported to alter responses to preconditioning.

Tang et al. found that the effects of PreC were lost in an in vivo rabbit model of hypercholesterolemia, owing to impaired upregulation of tetrahydrobiopterin (BH4), which is essential for inducible nitric oxide (NO) synthase[254]. However, our group in a study looking at the effects of both PreC and PostC on infarct size in rabbits found, contrary to the studies above, that the effects of PreC were preserved in hypercholesterolemic hearts, but lost in post-conditioned animals, using two different protocols for PostC[255].

Kupai et al. also noted the abrogation of the tissue-protective effects of PostC following a myocardial infarction in hypercholesterolemic rats, and sought to explain this by examining the protective role of early peroxynitrite-induced nitrosative stress and the lack of this mechanism in the hypercholesterolemic heart[256]. Others, however, have disagreed with these findings, suggesting that the effects of PostC, as well as PreC, were preserved in isolated rabbit hearts through the activation of the adenosine A1 receptor and K_{ATP} channels[257] .

In light of the loss of NO bioavailability and increased formation of peroxynitrite in hypercholesterolemic models, the effects of myocardial matrix metalloproteinase (MMP)-2 have also been examined. Isolated rat hearts from cholesterol fed Wistar rats were used to show that the protective inhibition of MMP-2 that is generated by IPC is blocked in hypercholesterolemic animals, and that a reduction in infarct size can be produced using an MMP inhibitor in non-preconditioned hearts[258]. More recently, the effect of PreC on cardiac gene expression has been examined, specifically looking at genes involved in NO and free radical signalling and in the mevalonate pathway, through which statins exert their effect[259].

Other factors proposed in the loss of the cardioprotective benefit of PreC include heat shock protein (HSP)-70, which is downregulated post-transcriptionally in hypercholesterolemia[260], and caspase-3, the activation of which is increased in hypercholesterolemic, ischemic rabbit myocardium and which has a central role in cell apoptosis[261].

3.2. Diabetes

Although some animal studies report that diabetes is not a barrier to PreC[262], the majority report that the diabetic heart[263] and the acutely hyperglycemic heart[264] are resistant to PreC. Several pathways are implicated in this lack of response, including impaired phosphorylation of PI3K-Akt, decreased generation of NO and eNOS, abnormal ERK1/2 activity, K_{ATP} dysfunction, and reduced release of calcitonin gene-related peptide (CGRP)[265]. Tsang et al. reported that, in the Goto-Kakizaki rat model of diabetes (a non-obese Wistar rat substrain that develops early type 2 diabetes), hearts could be preconditioned, but had a higher threshold for preconditioning. The diabetic animals required three cycles of PreC to achieve significant tissue protection following ischemic insult, whereas the non-diabetic controls required only one cycle[254].

The reduced efficacy of PreC in the diabetic heart might be due to alterations to the components of the PreC signal transduction pathway; for example, reduced Akt phosphorylation[254], mitochondrial K_{ATP} channel dysfunction[266], activation of GSK-3β[267] and reduced NO availability[268].

Recently it has been reported that hyperglycemia abrogates the infarct limiting effects of PostC by impairing AdipoR1/Caveolin-3/STAT3 signaling in diabetic rats[269]. This is in line with previous observations, which implicated a mitochondria dysfunction in terms of mPTP opening in the diabetic heart[270].

3.3. Hypertension and left ventricular hypertrophy

Many animal studies have attempted to investigate whether the hypertrophied heart responds to ischemic conditioning, with differences being reported in the models, methods and results. Some authors initially reported that hypertensive rat hearts could be preconditioned: Boutros and Wang demonstrated a preserved response to PreC in an isolated heart model from spontaneously hypertensive rats (SHRs)[271], whereas others demonstrated in an in vivo model of myocardial infarction that rats who developed left ventricular hypertrophy (LVH) in response to saline and a deoxycorticosterone-acetate hypertensive diet still benefited from PreC[272]. However, a subsequent study reported that hypertension abolished the cardioprotective effects of PreC in genetically hypertensive rats[273].

As with PreC, the effects of PostC in the hypertrophied myocardium demonstrate conflicting results, depending on the model of LVH used. Isolated SHR hearts display a reduced response to the cardioprotective effects of PostC[274], with a loss of GSK-3β phosphorylation as the likely mechanism[275]. Isolated, hypertrophied rat hearts, produced by chronic nandrolone treatment, were also found to be resistant to PostC[274]. Although the investigators did not find any difference in GSK-3β phosphorylation, they did note reduced Akt phosphorylation in the hypertrophied group. Conversely, when chronic treatment with angiotensin II was used to develop a model of LVH in rats[276], the beneficial effects of PostC were preserved. One explanation for the differing results observed in these different disease models is that SHRs are also known to have dyslipidemia and impaired glucose tolerance, making their failure to respond to PostC difficult to attribute entirely to hypertension.

3.4. Obesity

Many investigations have reported the metabolic that PreC is negatively affected by obesity. Evidence for this comes from work that reported that, although both obese and lean insulinresistant rats were resistant to PreC, post-ischemic recovery was impaired in obese animals compared with lean animals[277].

Lack of cardioprotection by PostC has also been demonstrated in ob/ob leptin-deficient mice, through a reduction in phosphorylation in members of the RISK pathway[278]. Similarly, in rats with the metabolic syndrome, a lack of GSK-3β and ERK phosphorylation have been linked to their inability to respond to PostC[279].

3.5. Senescence

The effects of preconditioning on the aged heart were investigated early in the quest to better understand this cardioprotective phenomenon. In 1996, isolated rat hearts were used to demonstrate that the effects of PreC were lost in the ageing heart, through a reduction in norepinephrine and α-adrenergic receptor activation in response to PreC[280], or due to the reduced translocation of protein kinase C[281].

These results have been substantiated in similar studies in rats, which have shown that the beneficial effects of PreC on the morphological consequences of ischemia were also lost in older rats[282, 283]. Furthermore, as with diabetes, the effects of ageing in 'middle aged' rats could be overcome by additional cycles of PreC[284].

Conversely, a study of isolated hearts from older rabbits reported no loss in the efficacy of PreC[285], although there was criticism that the animals used were not sufficiently aged to represent a true model of senescence[286]. Equally, a similar study of senescent sheep found that PreC-induced myocardial infarct size reduction was well preserved despite age[287].

Ageing does seem to reduce the beneficial effects of PostC. In senescent mice, the protective effects of PostC on myocardial ischemia are attenuated, with a proposed explanation being a decrease in ERK phosphorylation and an increase in mitogen-activated protein kinase phosphatase-1 (MKP-1) expression in the aged heart[288]. Furthermore, the response to PostC in the ageing mouse heart seems to depend on the protocol of PostC used, and a deficiency in STAT-3 associated with ageing might contribute to the dampened response[191].

3.6. Exposure to cigarette smoke

Smoking active or passive is an established risk factor for coronary heart disease (CHD)[289]. Smoking can be considered as a confounder which worseness myocardial responses to ischemia/reperfusion. Inhalation of smoke ingredients and direct nicotine exposure during cigarette smoking cause constriction of epicardial and resistance vessels[290], impair endothelium-dependent dilatation[291] and deteriorate the elastic properties of vessels in humans[292]. All these unfavorable effects are involved in the development of atherosclerosis, arterial hypertension and left ventricular hypertrophy. To date, there are no published studies designed to investigate the effects of exposure to cigarette smoke and its sequelae on conditioning. Nicotine exerts detrimental effects on the myocardium in the context of ischemia. Nicotine alters the balance of thromboxane/prostacyclin and aggravates myocardial ischemia, as judged by creatine phosphokinase and troponin T release. In addition, it

exacerbates postischemic contractile dysfunction of 'stunned' myocardium, and environmental tobacco smoke increases myocardial infarct size[293-296]. Additional studies suggest that smoking increases oxidative stress and causes cardiac remodeling[297], and exposure of cardiomyocytes to cigarette smoke causes toxicity and increases susceptibility to mPTP opening[298].

However, a direct effect of cigarette smoke exposure on conditioning mechanisms, which could explain the effects of cardioprotective strategies in smokers is not available.

Aim

In the present thesis, we sought to evaluate the role and the underlying molecular mechanisms of endogenous produced or exogenous administrated gasotransmitters, NO and H_2S, on

i) the pathophysiology of myocardial I-R injury;

ii) PostC mimicking effects in myocardial infarction and;

iii) the physiological role of these two gaseous molecules in cigarette smoking.

In brief we sought to examine:

1. The possible correlation of the endogenous production of NO by eNOS and its phosphorylation status with cardiomyocyte survival and to further investigate the contribution of the novel kinase PYK2 in the regulation of eNOS during myocardial infarction in vitro and in vivo.

2. Whether acute low dose exogenous administrated nitroglycerin, as a potent NO donor, mimicks ischemic postconditioning in in vivo models of myocardial ischemia-reperfusion injury. Furthermore, we goal to elucidate the underlying molecular mechanisms conferring to cardioprotection with focus on the endogenous NO pathway.

3. Whether exogenous administrated NaHS, as an H_2S donor, mimicks postconditioning in in vivo models of myocardial ischemia-reperfusion injury. Furthermore, we goal to elucidate the underlying molecular mechanisms conferring to cardioprotection focusing on eNOS/ PKG/cGMP/PLN pathway

4. Evaluation of cigarette smoke as a new co-morbidity on intracellular myocardial signaling, infarct size after ischemia- reperfusion injury and the potential interference with ischemic conditioning. Furthermore, we goal to emphasize on the role of endogenous NO and H_2S in the mechanisms underlying cigarette smoking effects on myocardial infarction.

In this context four separate studies have been performed.

Herein, I am presenting you the manuscripts with slight differences concerning:

Experimental study I: submitted on Cardiovascular Research Journal

Experimental study II: submitted on Circulation Journal

Experimental study III: published on Cardiovascular Research 2015 Jun 1;106(3):432-42. doi: 10.1093/cvr/cvv129

Experimental study IV: under revision on Am Journal of Physiology Heart and Circulation Physiology

Experimental Study I:

Tyrosine phosphorylation of eNOS regulates myocardial survival after an ischemic insult: role of PYK2.

1.1. Abstract

Aim: Reperfusion of the myocardium following sustained ischemia protects the tissue from more extensive necrotic death, but is associated with paradoxical injury, compromising the overall benefit of reinstating blood supply. Although endothelial nitric oxide (NO) synthase (eNOS) is known to play an important protective role, the molecular mechanisms regulating eNOS activity during ischemia-reperfusion injury in the heart are incompletely understood. eNOS is a substrate for several kinases that positively or negatively affect its enzymatic activity. Akt phosphorylates eNOS on S1177 increasing its enzymatic activity, while proline rich tyrosine kinase 2 (PYK2) phosphorylates eNOS on Y657 and eliminates NO output. Herein, we sought to correlate eNOS phosphorylation status with cardiomyocyte survival and to investigate the contribution of the PYK2/eNOS axis to myocardial infarct size.

Methods & Results: Oxidative stress injury (H_2O_2, 50μM) to differentiated H9c2 cardiomyocytes lead to an increase of PYK2 phosphorylation on its activator site (Y402). Exposure to H_2O_2 also resulted in an increase in eNOS phosphorylation on the inhibitory site Y656 and on the activator site S1176. Both H_2O_2–induced phosphorylation events were abolished by pharmacological inhibition or gene knockdown of PYK2. Activity assays demonstrated that Y657 exerts a dominant effect over S1176. In cell viability experiments, we observed that survival was augmented under either oxidative stress or oxygen-glucose deprivation injury when PYK2 is inhibited; this effect was reversed by inhibition of NO production. Our findings were confirmed *in vivo*; ischemia-reperfusion activated PYK2 early during reperfusion, leading to eNOS phosphorylation on Y656 and reduced NO output, as judged by the low tissue cGMP levels. Moreover, pharmacological blockade of PYK2 alleviated eNOS inhibition and prevented cardiac damage following ischemia-reperfusion in wild-type, but not in eNOS KO mice.

Conclusion: PYK2 is a pivotal regulator of eNOS function in myocardial infarction and could serve as a new cardioprotective strategy.

1.2. Aim

Taken into consideration i) that the knowledge on eNOS regulation by phosphorylation is limited in the heart muscle (described in detail in section 5) and ii) the novel mechanism by which PYK 2 negatively regulates eNOS in aortic endothelium under shear stress (described in detail in section 5). The specific aim of the present study is to understand the involvement of the novel kinase PYK 2 in eNOS regulation in ischemia reperfusion injury of heart muscle. Better understanding of eNOS regulation by phosphorylation and the identification of the kinases involved in positive or negative regulation of the synthase, will serve as a novel therapeutic strategy for minimizing organ cell death under different conditions in which ischemia reperfusion injury plays a predominant role.

The present study will allow us to rigorously address the following questions and needs:

1. If oxidative stress induced injury and in which extent can induce PYK 2 activation in cardiomyocytes

2. If the observed activation of PYK2 regulates eNOS by altering the phosphorylation status and the ability to change NO bioavailability and to mediate NO derived signaling in cardiomyocytes

3. If inhibition of PYK2 increases cell viability under oxidative stress injury and hypoxia-reoxygenation induced injury through alleviating eNOS.

4. If in in vivo model of myocardial infarction the oxidative stress induced by ischemia or/and reperfusion can regulate PYK2-eNOS

5. If inhibition of PYK2 limits myocardial infarct size in an eNOS dependent manner.

1.3. Materials and Methods:

1.3.1. Chemicals

DMEM and 10% fetal bovine serum (FBS) were obtained from Gibco-Thermo Scientific (Waltham, MA USA. siRNAs against rat PYK2 (NM_017318 SASIRn0100044069) and scrambled negative control (Synonym: MISSION® siRNA Universal Negative Control) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The LDH cytotoxicity assay kit was purchased from Cayman Chemical (Lab Supplies-P. Galanis & Co, Greece). Lipofectamine™ RNAiMAX and Opti-MEM medium were obtained from Invitrogen, Thermo Scientific (Waltham, MA USA). The RNeasy Mini Kit was purchased from Qiagen GmbH (Dusseldorf, Germany). Real-time PCR was performed with the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems Ltd, London, UK). The QuickChange XL site-directed mutagenesis kit was purchased from Stratagene (Amsterdam, Netherlands). The cGMP kit was from Enzo Life Sciences GmbH (Lörrach, Germany). Protein G SepharoseTM 4 Fast Flow was from GE Healthcare (Upsala, Sweden). The following primary antibodies phospho PYK2 (Y402), phospho eNOS (S1176), phospho eNOS (T495), phospho Akt (S473), phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199), total PYK2, β-tubulin, and the goat anti-rabbit HRP antibody were from Cell Signaling Technology (Beverly, MA, USA). The phospho eNOS Y656 antibody was generated by Eurogentec (Searing, Belgium). The SuperSignal chemiluminescence kit were from Thermo Scientific Technologies (Waltham, MA USA). H_2O_2 was obtained from AppliChem GmbH (Darmstadt, Germany). All other reagents including NaCl, NaF, EDTA, EGTA, PMSF, PF-431396 and MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

1.3.2. Cell culture

The rat embryonic-heart derived H9c2 cell line was obtained from ATCC (CRL-1446™) (ATCC, LGC Standards, Middlesex, UK). H9c2 cells were cultured in Dulbecco's modified essential medium DMEM containing 25 mM D-glucose, 1 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% streptomycin (100 μg/ml), and 1% penicillin (100 U/ml) at pH 7.4 in a 5% CO_2 incubator at 37°C. For differentiation, H9c2 were seeded and allowed to grow to confluence. The medium was then replaced to DMEM containing 1% FBS with 10 nM all-*trans*-retinoic acid for 7 days. Culture of H9c2 myoblasts in low serum media and stimulation with 10 nM all-*trans*-retinoic acid for seven days resulted in the appearance of elongated cells connecting at irregular angles reminiscent of cells with a cardiac phenotype[299].

1.3.3. Transient transfection with siRNAs

H9c2 cells were seeded until 80% confluence. Differentiation protocol was followed. Transient transfection of siRNAs (100 nM) was performed using Lipofectamine™ RNAiMAX, according to the manufacturer's instructions. The transfection complex was diluted into Opti-MEM medium and added directly to the cells. After 24 hrs, the Opti-MEM was replaced with complete DMEM medium with 10% FBS for cell viability assays or with serum-free DMEM for biochemical studies. To test the efficacy of siRNA PYK2 gene knockdown, 48 hrs post-transfection, total RNA was extracted and mRNA was reverse-transcribed into cDNA and analyzed by real-time PCR. Real-time PCR was performed with

primers specific for rat PYK2, eNOS and GAPDH. The expression levels of PYK2 and eNOS were determined by normalizing to GAPDH.

1.3.4. Western Blot Analysis

H9c2 cells seeded in 6-well plates until confluence , further differentiated and treated as described above were washed twice with PBS and further lysed with lysis solution (1% Triton X100, 20 mM Tris pH 7.4-7.6, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Glycerolphosphatase, 1% SDS, 100 mM PMSF, supplemented with protease and phosphatase inhibitor cocktail(Sigma). For tissues, frozen ischemic samples were pulverized and homogenized with the lysis buffer. The lysates were centrifuged at 11,000 g for 15 min at 4 °C. The supernatants were collected and the protein concentration was determined based on the Lowry assay. The supernatant was mixed with a buffer containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenyl blue, 0.125 M Tris/HCl. The samples were then heated at 100^{o}C for 10 min and stored at -80^{o}C. An equal amount of protein was loaded in each well and then separated by sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4 °C with primary antibody. The following primary antibodies were used: phospho PYK2 (Y402), phospho eNOS (T495, Y657 and S1176), phospho Akt (S473), β-tubulin. Membranes were then incubated with secondary goat anti-rabbit HRP antibody for 2 h at room temperature and developed using the Supersignal chemiluminesence ECL Western Blotting Detection Reagents. Relative densitometry was determined using a computerized software package (NIH Image) and the values for phosphorylated were normalized to the values for total proteins respectively.

1.3.5. Immunoprecipitation

Lysates containing 250 μg of protein were incubated with anti PYK2 antibody and protein G-conjugated sepharose beads overnight at 4°C. To reduce nonspecific binding, the protein G beads were preincubated with purified bovine serum albumine for 2 h at 4 °C and washed twice with fresh lysis buffer before adding to the samples. Next day, the beads were washed 5 times with lysis buffer and immunoprecipitated proteins were subjected to SDS-PAGE and transfered to PVDF memebrane. The membranes were then blocked and incubated with the phospho PI3K p85 (Y458) or phospho Akt (Ser473) and PYK2 primary antibodies and goat anti-rabbit HRP antibody. Immunoreactive proteins were detected using the SuperSignal chemiluminescence kit.

1.3.6. eNOS activity measurements

Myc-tagged human eNOS cDNA (GenBank accession no. NM_000603) cloned in pcDNA3.1myc/His was used. eNOS mutants (Y657D and Y657D/S1177D) were generated using standard site-directed mutagenesis procedures according to the manufactures' instruction (QuickChange XL site-directed mutagenesis kit). For the transfection experiments, HEK293 cells were plated in 12-well plates, grown overnight, and transfected with the indicated plasmids, using a total of 2 μg DNA and 4 μL of Lipofectamine transfection reagent per well in Opti-MEM medium. After 24 hours cells were used for LC-MS and western blot

measurements. For western blot studies, cells were starved for 4h in serum free medium with addition of 0.1% of BSA. For NO measurements, HEK cells were treated either with vehicle ddH2O, or sepiapterin 10 μmol for 2h before ionomycin 100nmol treatment for 10 minutes. Arginine depletion was performed by addition of SILAC medium for 2 hours. Heavy labeled Arg (13C6, 15N4) was added for 20 minutes before collection for the basal activity or stimulation with sepiapterin and ionomycin for the stimulated activity. Cells were immediately emerged in liquid nitrogen and 85% HPLC grade MeOH was added for cells disruption. The lysates were centrifuged in 12000xg for 15 minutes in 4^0C. 50μl of supernatant was mixed with 50μl of MeOH containing 1mg/ml glutamine as internal standard. The mixture was used for LC-MS measurements.

1.3.7. eNOS activity arginine/citrulline measurement

Conversion of heavy labeled Arg (13C6, 15N4) to heavy Cit (13C6, 15N3) were analysed by hydrophilic interaction chromatography coupled to mass spectrometry. Liquid chromatography separation was performed on an Agilent 1290 Infinity pump system (Agilent, Waldbronn, Germany), using a Phenomenex (Aschaffenburg, Germany) Kinetex HILIC column (100 mm × 2.1 mm, 2.6 μm) at a column oven temperature of 35 °C. The gradient between solvent A (10 mM amonium formate) and solvent B (acetonitrile + 0.15% formic acid) was as follows: 0.0 to 0.5 min 10% A, than increase to 85% A from 0.5 to 6 min, than increase to 98% A to 6.1 min, which was held until 13.6 min. Subsequently, column was reconditioned with 90% A for 4.4 min. The flow rate was set to 350 μl/min and the injection volume was 2.5 μl. Mass spectrometry was performed using a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany) with electro spray ionization at 300°C with 3500 V in positive mode. MS parameters were set to CUR 30 psi, GS1 50 psi, and GS2 40 psi. Data acquisition and instrument control were managed through the software Analyst 1.6.2. Peak integration, data processing, and analyte quantification were performed using MultiQuant 3.0 (Sciex, Darmstadt, Germany). Area under the peak was used as the quantitative measurement. The specific MRM transition for every compound was normalized to appropriated isotope labeled internal standards.

1.3.8. In vitro oxidative stress and oxygen-glucose deprivation/recovery

To induce oxidative stress injury H9c2 cells ($1.5x10^4$ per well) were differentiated in 96-well plates. The cells were treated with 500μM H_2O_2 in serum-free DMEM for 12h in a 5% CO_2 incubator at 37°C. In the oxygen glucose deprivation/ recovery (OGD/R) assay, differentiated H9c2 cells in 96 well plates were rinsed twice with PBS and incubated in glucose-free DMEM and subsequently placed in an anaerobic chamber containing a mixture of 95% N_2 and 5% CO_2 at 37°C for 24h. Following OGD/R, glucose was added to normal levels (final concentration: 4.5 $mg \cdot mL^{-1}$), and cells were incubated under normal growth conditions (95% air and 5% CO_2) for an additional 12 h.

1.3.9. MTT measurement

H9c2 cells were seeded in 96-well plates at $1.5x10^4$ per well in growth medium. Following oxidative stress injury or OGD/R; cell survival was assessed in differentiated H9c2 cells by using the conversion of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

to formazan. Cells were incubated with MTT at a final concentration of 0.5 mg/ml, for 2 h at 37°C. The formazan formed was dissolved in solubilization solution (10% Triton-X 100 in acidic 0.1N HCl isopropanol); subsequently, absorbance was measured at 595 nm with a background correction at 750 nm using a microplate reader.

1.3.10. LDH measurement

Lactate dehydrogenase (LDH) release was used to detect cytotoxicity/cell death using a commercially available kit. In brief, supernatant medium was collected and centrifuged at 400xg for 5 minutes. Cell supernatant (100 μl) was transferred to a new 96-well assay plate and 100 μl of reaction solution- containing 1 mM NAD+, 85 mM Lactic acid, 0.5mM INT and LDH diaphorase- was added to each well. The plate was incubated for 30 minutes at 37^{o}C with gentle shaking. Absorbance was read at 490 nm with a plate reader.

1.3.11. cGMP enzyme immunoassay

H9c2 cells were washed twice with Hank's balanced salt solution (HBSS) and incubated in HBSS in the presence of IBMX (1 mM) for 15min. After the indicated treatment, media were then aspirated and 125 μl of 0.1 N HCl were added into each well to extract cGMP. After 30 min, HCl extracts were collected and centrifuged at 600 g for 10 min to remove debris. The supernatants were directly analyzed for cGMP by a commercially available EIA kit following the manufacturer's instructions. After HCl extracts had been removed, 100 μl of 0.5 M NaOH were added to each well, and total protein was determined by the Lowry method. For tissue samples, frozen ischemic tissue was pulverized. Powdered samples from myocardial ischemic tissue were treated with 0.1N HCl (1:5 vol/w) to extract cGMP. cGMP content was measured in the lysates using enzyme immunoassay. Results were expressed as pmol cGMP/mg protein.

1.3.12. Animals

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals; experimental protocols were approved by the Ethical Committee of the Prefecture of Athens. Animals received the usual laboratory diet. In the present study male mice C57BL/6 or male eNOS KO mice were used.

1.3.12.1. Surgical Procedures

Murine *in vivo* Model of Ischemia-Reperfusion Injury

Male mice 10-12 weeks old were anesthetized by intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.01 mL/g, final concentrations of ketamine, xylazine and atropine 10 mg/mL, 2 mg/mL, 0,06 mg/kg respectively). A tracheotomy was performed for artificial respiration at 120-150 breaths/min and PIP 2.0 (0.2 mL tidal volume) (Flexivent rodent ventilator, Scireq, Montreal, Ontario, Canada). Following anesthesia the left carotid artery was revealed and catheterized by the use of a 18G catheter for rabbits model and a 29G catheter for mice model. Mean arterial blood pressure was monitored through a fluid filled transducer connected to a BIO AMP amplifier and indicated electrocardiogram recording was performed by a Lead I ECG recording with PowerLab 4.0 (ADInstruments, UK). Recordings

were analyzed by LabChart 7.0 software. A thoracotomy was then performed between the fourth and fifth ribs and the pericardium carefully retracted to visualize the LAD, which was ligated using a 8-0 prolene monofilament polypropylene suture placed 1 mm below the tip of the left auricle. The heart was allowed to stabilize for 15 minutes prior to ligation to induce ischemia. After the ischemic period, the ligature was released allowed reperfusion of the myocardium. Throughout experiments, body temperature was maintained at $37^oC \pm 0.5^oC$ by way of a heating pad and monitored via a thermocouple inserted rectally. After reperfusion hearts were rapidly excised from mice and directly cannulated and washed with 2.5 ml saline-heparin 1% for blood removal. 5 ml of 1% TTC phosphate buffer 37^0 C were infused via the cannula into the coronary circulation followed by incubation of the myocardium for 5 minutes in the same buffer; 2.5 ml of 1% Evans blue, diluted in distilled water was then infused into the heart. Hearts were kept in -20^0C for 24h and then sliced in 1mm sections parallel to the atrio-ventricular groove, and then fixed in 4% formaldehyde overnight. Slices were then compressed between glass plates 1 mm apart and photographed with Cannon Powershot A620 Digital Camera through Zeiss 459300 microscope and measured with the Scion Image program. The areas of myocardial tissue at risk and infracted were automatically transformed into volumes. Infarct and risk area volumes were expressed in cm^3 and the percent of infarct to risk area ratio (%I/R) is calculated.

1.3.12.2. Experimental protocol

Mice were sham operated or subjected to 30 minutes LAD occlusion followed by reperfusion for 3, 10 or 30 minutes. At the end of the experiment, tissue samples from the upper part of the left ventricle (sham or ischemic part) were excised and snap frozen in liquid nitrogen. In animals treated with the pharmacological inhibitor of PYK2, PF-431396 was administrated at 5μg/g PF-431396 in 2% DMSO (100μl) IV 10 minutes prior to the ischemic insult. In the control group, vehicle (100μl) containing a 2% DMSO in water for injection was administrated IV 10 minutes prior to the ischemic insults.

In a second series of experiments, mice were subjected to 30 minutes regional ischemia of the myocardium, followed by 2 hours of reperfusion and were randomized into 5 groups as follows. 1) Control group (n=8): administration of vehicle (water for injection containing 2% DMSO [100μl] IV 10 minutes prior to the ischemic insult); 2) PF-431396 pretreatment group (n=9): administration of 5μg/g PF-431396 (dissolved in DMSO 2% in water for injection; (100μl) IV 10 minutes prior to the ischemic insult; 3) PF-431396 post-treatment group (n=7): administration of 5μg/g PF-431396 (100μl iv 10 minutes prior to reperfusion); 4) eNOS KO group (n=6): administration of vehicle 10 minutes prior to the ischemic insult; 5) eNOS KO + PF-431396 group (n=7): administration of 5μg/g PF-431396 IV 10 minutes prior to the ischemic insult.

1.4. Results

1.4.1. PYK2 increases phosphorylation of eNOS on S1176 and Y657 in cardiomyocytes exposed to H_2O_2

Exposure of H9c2 differentiated cardiomyocytes to H_2O_2 for 10min resulted in increased phosphorylation of PYK2 on the autophosphorylation Y402 activator site (Fig. 1A). In addition, increased phosphorylation of both Y656 and S1176 on eNOS was observed (Fig. 1B, 1C).

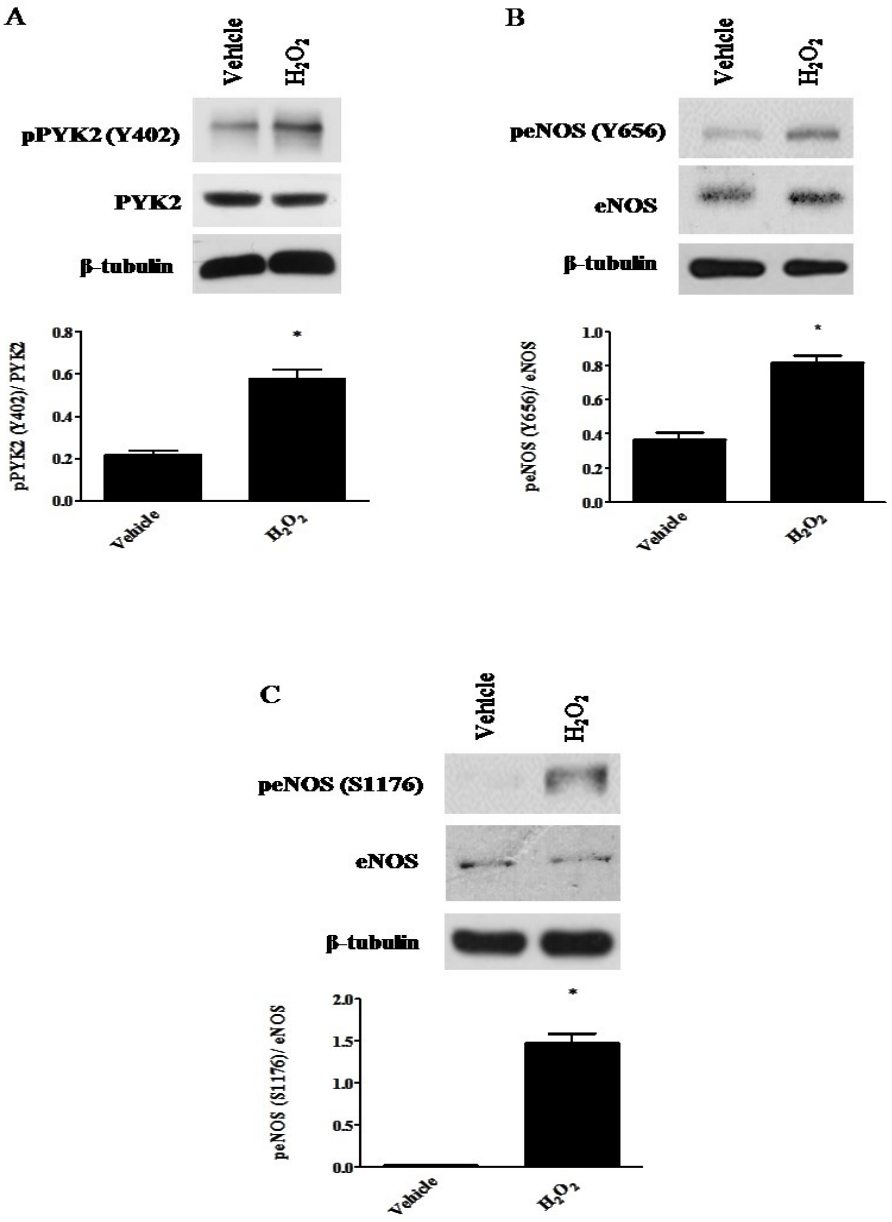


Figure 1: Exposure of cardiomyocytes to H_2O_2 increases PYK2 and eNOS phosphorylation. Differentiated H9c2 were treated with 50μM H_2O_2 for 10 minutes, lysed and proteins subjected to SDS-PAGE. Representative western blots and densitometric analysis of A. phospho-PYK2 (Y402); B. phospho-eNOS (Y656); C. phospho-eNOS (S1176). Results were normalized for total protein levels. β tubulin is also presented as reference protein. n=6; *p<0.05 vs vehicle.

Silencing of PYK2 was documented in siRNA-treated cells, which exhibited reduced mRNA (Fig. 2A) and protein (Fig. 2B) levels of PYK2. PYK2 knockdown did not affect eNOS mRNA (Fig. 2C) /protein levels (Fig 2D).

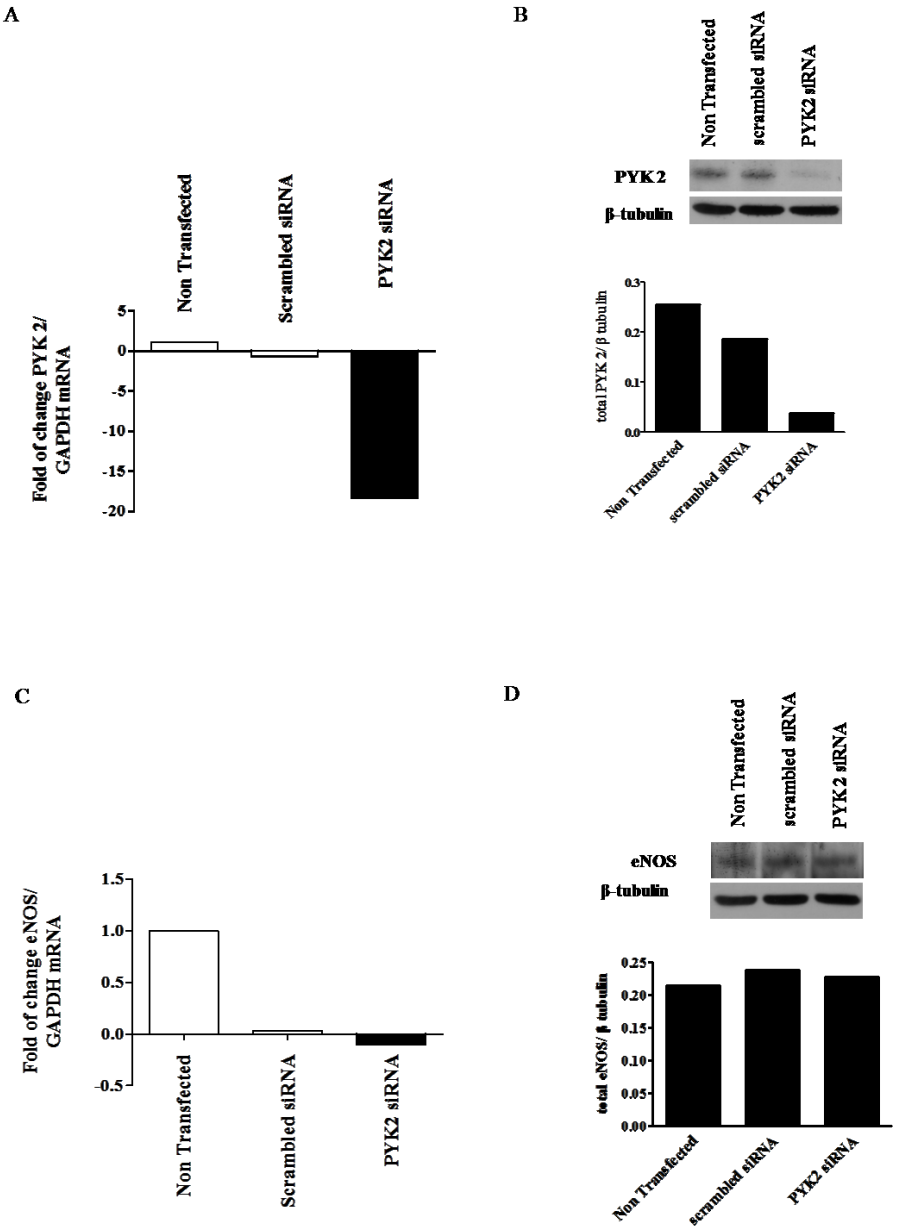
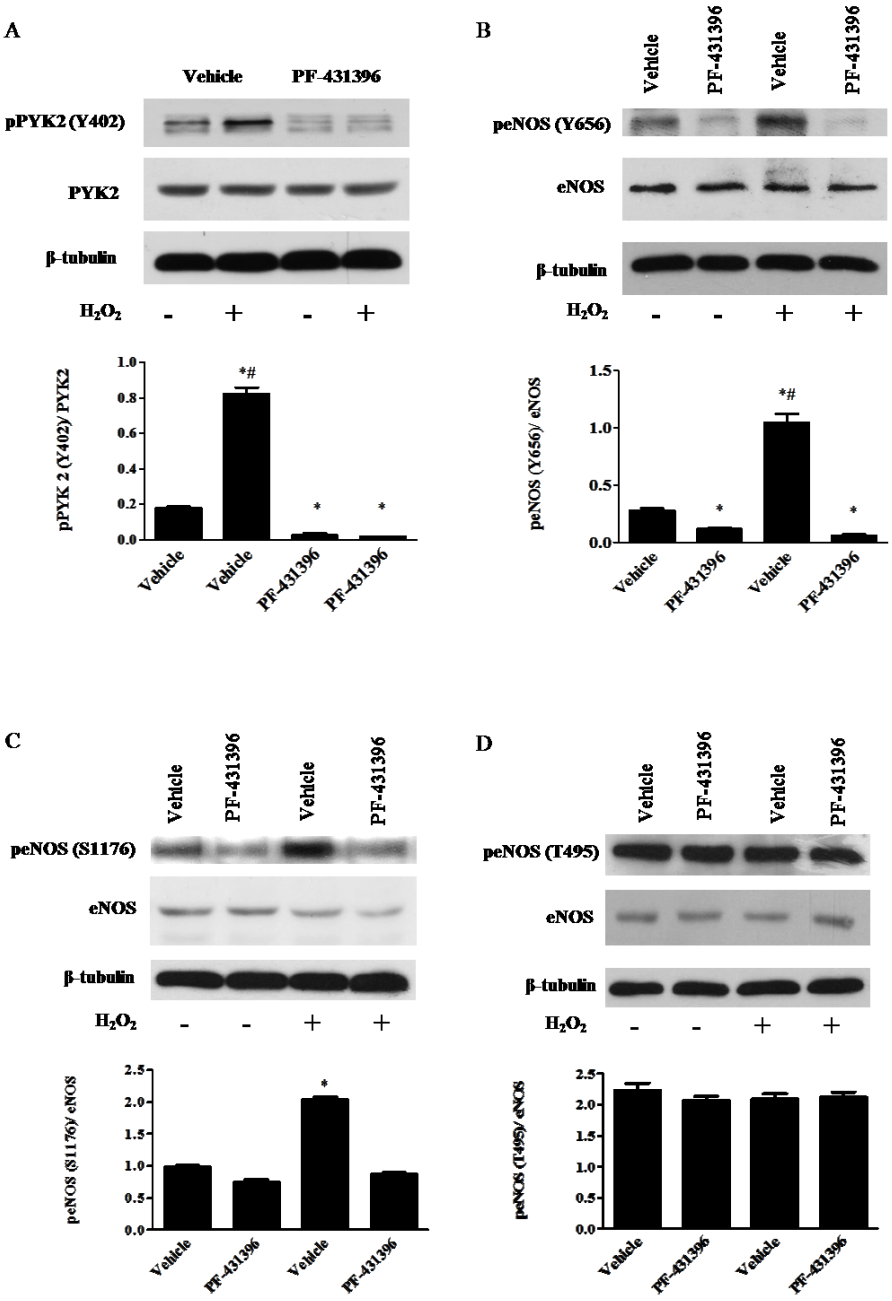


Figure 2: siRNA reduces PYK 2 levels but does not affect eNOS protein levels. mRNA levels of (A) PYK2 and (C) eNOS and representative western blot and densidometric analysis of (B) PYK2/ β-tubulin, (D) eNOS/ β-tubulin of H9c2 lysates. Cells were transfected for 48h with the indicated siRNAs using a Lipofectamine RNAimax mixture, as described in the supplementary methods.

Pharmacological inhibition of PYK2 by PF-431396 reduced the H_2O_2-triggered Y402 phosphorylation of the kinase (Fig. 3A). Moreover, inhibition of the kinase, abolished the phosphorylation induced by oxidative stress injury on both eNOS Y656 (Fig.3B) and S1176 (Fig.3C), whilst T495 phosphorylation was unaffected (Fig.3D).



To confirm the results obtained with the pharmacological PYK2 inhibitor, we used a siRNA knockdown approach. While PYK2 and eNOS phosphorylation on Y657 and S1176 were induced after exposure to H_2O_2 in non-transfected or scrambled siRNA-transfected cell, such treatment was ineffective in enhancing eNOS phosphorylation in cells in which PYK2 expression had been silenced (Fig.3E-G).

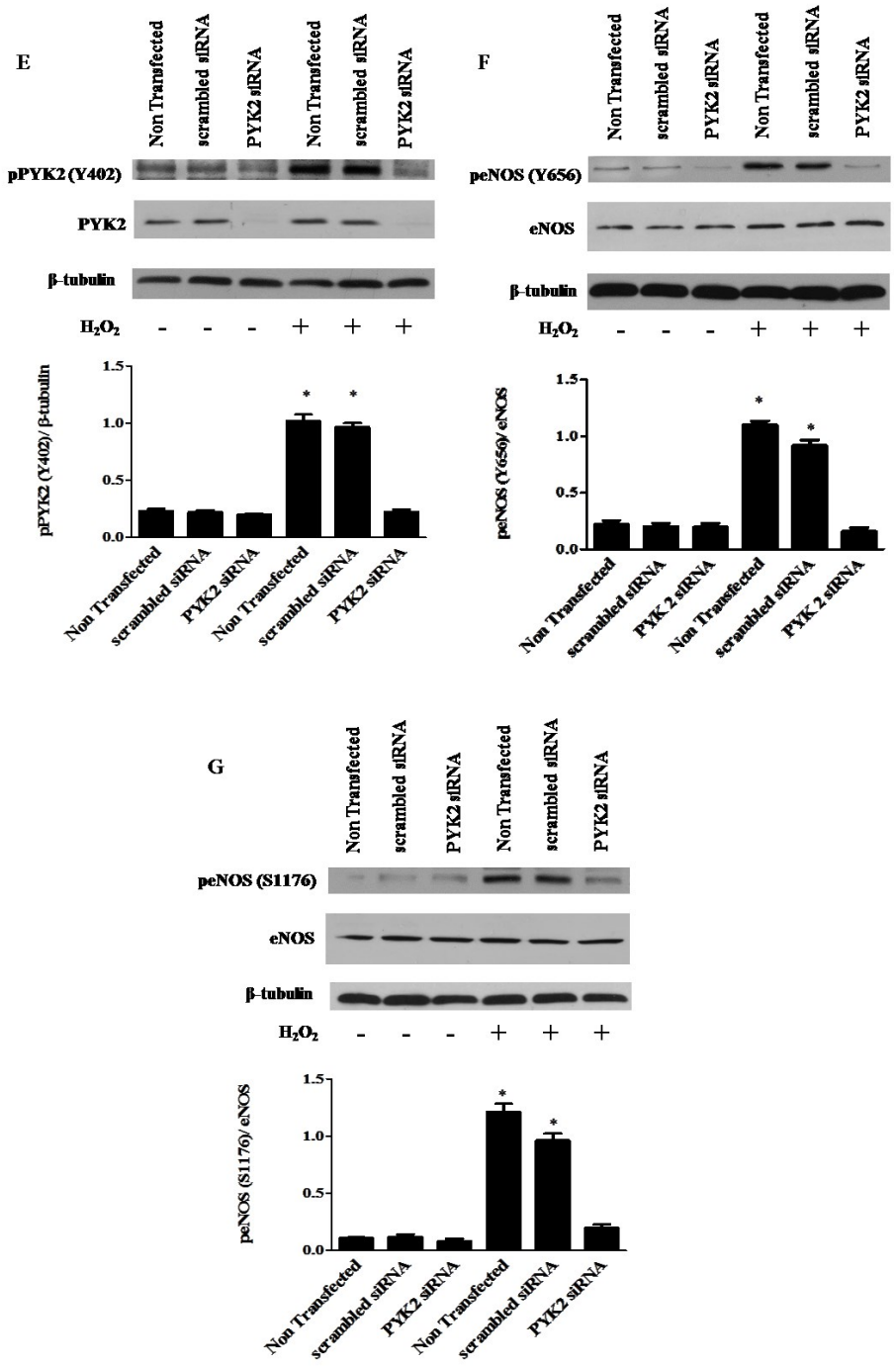


Figure 3: Inhibition of PYK2 reduces H_2O_2-induced eNOS phosphorylation. Differentiated H9c2 were treated with 50μM H_2O_2 for 10 minutes; to inhibit PYK2 cells were pretreated with 5μM PF-431396 for 45 min. To knockdown PYK2 expression cells were transfected with a PYK2 siRNA as described in the Methods section. Representative western blot and densidometric analysis of phospho-PYK2 (Y402) A, E; phospho-eNOS (Y656) B, F.; phospho-eNOS (S1176) C, G.; phospho-eNOS (T495) D. Results were normalized total protein levels. β tubulin is presented as reference protein. n=6; *p<0.05 vs vehicle (A, B, C), *p<0.05 vs NT (E, F, G), #p<0.05 vs PF-431396 (A, B). NT (non-transfected)

1.4.2. Increased S1176 phosphorylation of eNOS is PYK2-PI3K-Akt mediated

As PYK2 is a tyrosine kinase, the observation that PYK2 enhanced S1176 eNOS phosphorylation suggested the existence of an intermediate kinase that would lie downstream of PYK2 and could phosphorylate S1176. H_2O_2 treatment increased Akt phosphorylation in H9c2 cells (Fig. 4A). This effect was inhibited by either pharmacological inhibition of PYK2 (Fig. 4B) or PYK2 silencing (Fig. 4C).

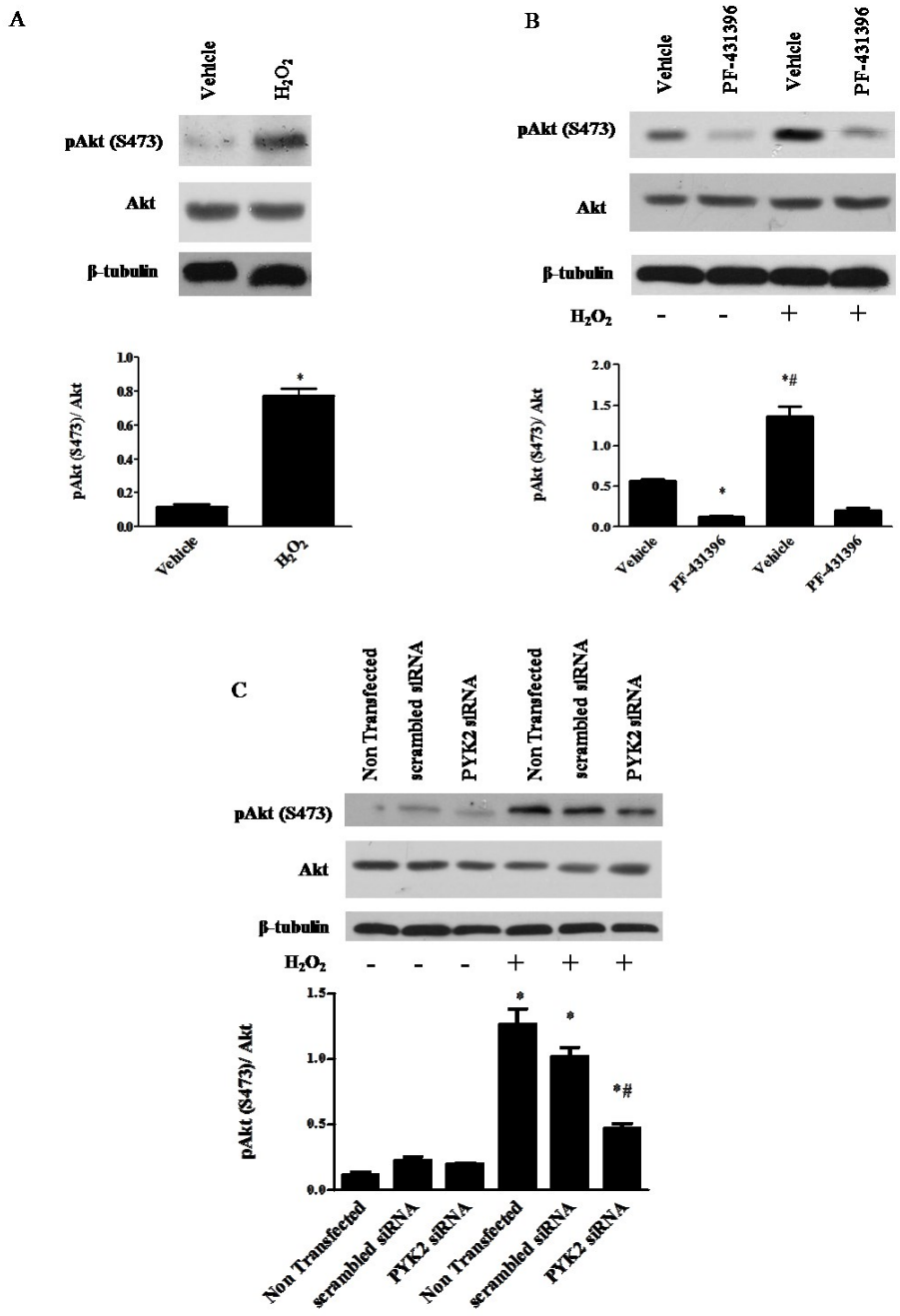


Figure 4: Oxidative stress (H_2O_2) activates Akt in a PYK2-dependent manner in cultured cardiomyocytes. Differentiated H9c2 were treated with 50μM H_2O_2 for 10 minutes; to inhibit PYK2 cells were pretreated with 5μM PF-431396 for 45 min. To knockdown PYK2 expression cells were transfected with a PYK2 siRNA, as described in the Methods section. Representative western blot and densidometric analysis of phospho-Akt (S473) normalized

for total Akt content.β tubulin is presented as reference protein; n=6 *p<0.05 vs vehicle (A, B), p<0.05 vs NT (C), #p<0.05 vs PF-431396 (B), #p<0.05 vs NT+H_2O_2 (C). NT (non transfected)

Moreover, in immunoprecipitation experiments we observed that the PI-3K subunit p85 was phosphorylated on Y458 and associated with PYK2 in H_2O_2-treated cells (Fig. 5A). Activation of PI-3K, as assessed by p-p85, was abolished by inhibiting the activity (Fig 5B) or silencing PYK2 (Fig.5C).

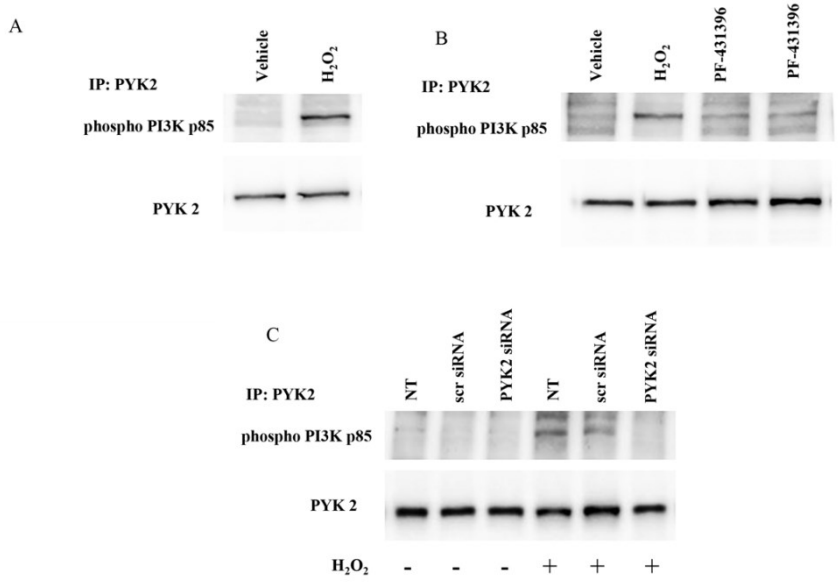


Figure 5: PYK 2 activates PI3K following H_2O_2 treatment. PYK2 was immunoprecipitated and precipitates were blotted for the phosphorylated form of the p85 subunit. Determination of PYK2 levels demonstrates equal amounts of immunoprecipitated protein. Cells treated with 50μM H_2O_2 for 10 minutes and pretreated with PF-431396 5μM or vehicle for 45 min. For transfections, cells were processed as in Fig.4. Blots shown are representative of experiments performed three times.

1.4.3. The dominant phosphorylation site determining eNOS activity is Y657

Although, Y657 (Y656 for mouse eNOS) phosphorylation is known to inhibit eNOS activity, it is unknown whether the negative impact of Y657 phosphorylation on eNOS activity prevails over the positive effect of S1177 when both residues are phosphorylated. We generated human eNOS mutants for Y657 and S1177 by converting the residues to the phosphomimetic aspartic acid (D). HEK293 cells were transfected with empty vector, wild-type eNOS, eNOS Y657D and the Y567D/S1177D double mutant; all mutants exhibited equal eNOS expression (Fig6. A). Cells transfected with wild-type eNOS showed an increased ability to convert arginine to citrulline. Y657D eNOS was completely inactive, in line with our previous observations (Fig. 6B). The double phosphomimetic Y657D/S1177D mutant, unlike what has been shown for the S1177D that expresses higher activity than wild-type eNOS [300-302], exhibited no basal or stimulated eNOS activity. Similar results were

obtained by measuring NO_x in the supernatant of transfected cells, as a readout for eNOS activity (n=2) (Fig. 6C).

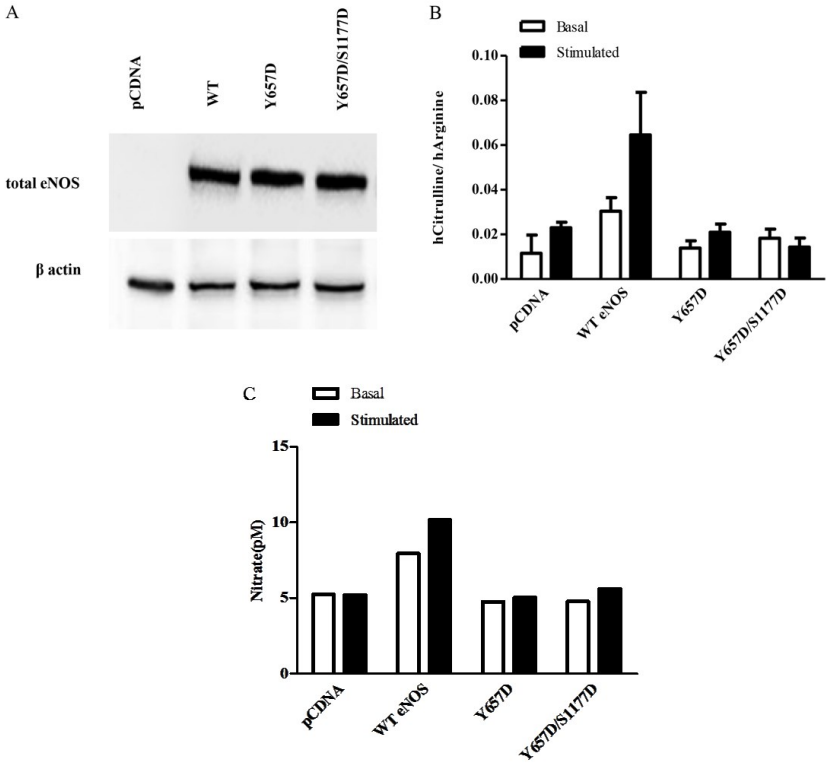


Figure 6: Phosphorylation of Y657 dominates over phosphorylation of S1177, determining NO production. A. Representative western blot of eNOS expression levels normalized for β-actin in HEK239 lysates transfected with the eNOS mutants. B. eNOS activity was determined from the heavy citrulline/heavy arginine ratio for each mutant under basal or stimulated conditions by LC-MS. C. Chemiluminesence assessment of eNOS actity in HEK239 transfected cells as indicated. For stimulated eNOS activity, 10μM sepiapterin and 100nmol ionomycin were added. n=4-6.

1.4.4. PYK2 inhibition or knockdown rescues cardiomyocytes from oxidative stress injury or OGD/R by alleviating eNOS inhibition

Oxidative stress injury of H9c2 cells following exposure to 500μM H_2O_2 resulted in reduced formation of formazan after incubation with MTT (Fig 7A). In addition, cells exposed to 500μM H_2O_2 exhibited increased LDH release (Fig 7B). Treatment with L-NAME or PF-431396 under basal conditions did not alter the behavior of cells in the MTT assay or alter LDH release. However, pharmacological inhibition of PYK2 in the context of oxidative stress injury significantly reduced cell toxicity (Fig.7A, 7B). This beneficial effect of PF-431396 was reversed upon inhibition of eNOS by L-NAME (Fig. 7A,7B).

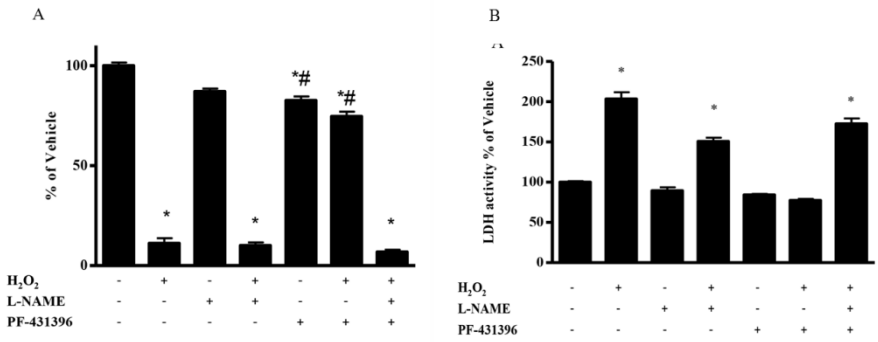


Figure 7: Inhibition of PYK2 promotes cell viability after injurious insults in vitro. Differentiated H9c2 cells were incubated with the indicated treatment and then subjected to 12h H_2O_2 500μM exposure. At the end of the incubation cell viability was assessed by the (A) MTT assay and (B) LDH activity assay. Treatments with PF-431396 and L-NAME were 5μM for 45 minutes and 100μM for 40 minutes prior to the assays; n=4-8, *p<0.05 vs vehicle (A,B), # p<0.05 vs H_2O_2 (A). PYK2 was inhibited with PF-431396 (5μM for 45 minutes).

The results were replicated by silencing PYK2; reducing expression of the kinase rendered the cells less susceptible to injury. This beneficial effect was reversed by addition of L-NAME (Fig. 8A; 8B).

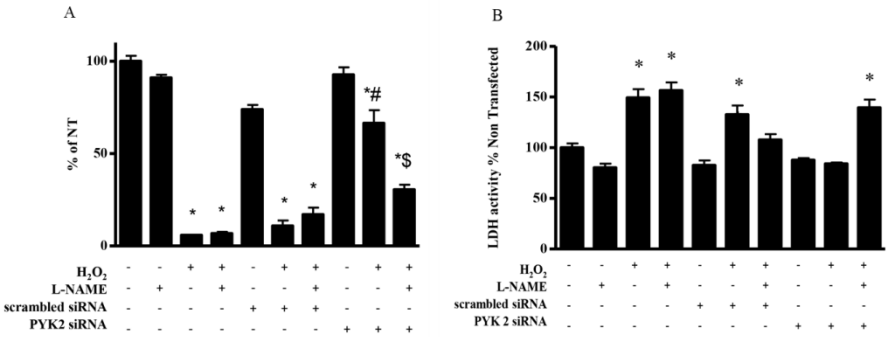


Figure 8: Silencing of PYK2 promotes cell viability after injurious insults in vitro. Differentiated H9c2 cells were transfected with siRNAs and then subjected to 12h H_2O_2 500μM exposure. At the end of the incubation cell viability was assessed by the (A) MTT assay and (B) LDH activity assay. Treatment with L-NAME was 5μM for 45 minutes and 100μM for 40 minutes prior to the assays; NT: non-transfected. n=4-8, *p<0.05 vs NT (A,B), # p<0.05 vs H_2O_2 , $ p<0.05 vs PYK2 siRNA+H_2O_2 (A). PYK2 was silenced for 48hr. NT (non transfected)

Additional evidence for the protective effects of PYK2 inhibition in H9c2 was provided in experiments in which H9c2 were subjected to oxygen/glucose deprivation (OGD) injury. Pharmacological inhibition of PYK2 (Fig. 9A; 9B) or PYK2 knockdown (Fig. 9C, 9D) resulted in increased cell survival, as assessed by MTT conversion. Similarly to what was

seen with H_2O_2, the beneficial effects of PYK2 inhibition in cells exposed to OGD were abrogated by L-NAME.

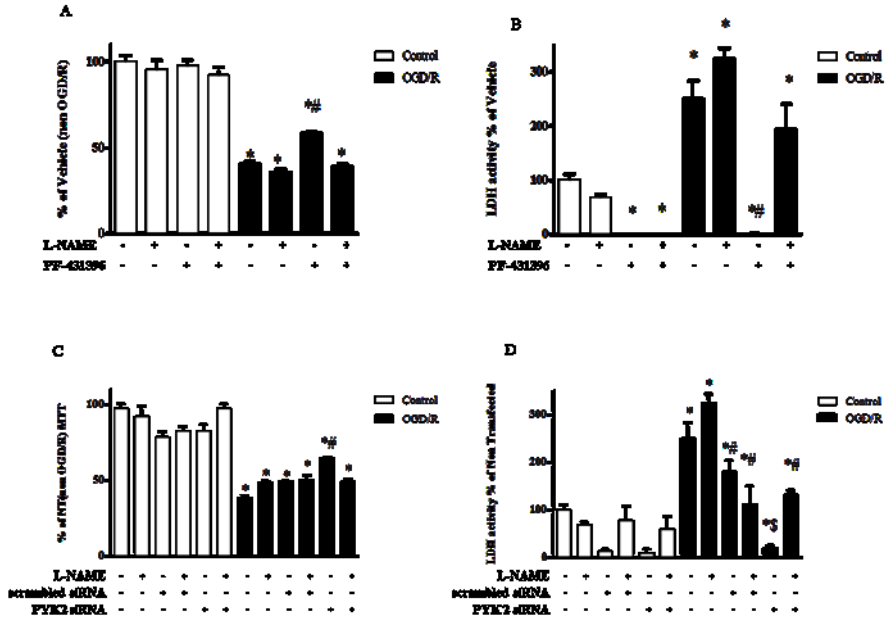


Figure 9: Inhibition or silencing of PYK2 promotes cell viability after injurious insults in vitro: Cells subjected to the indicated treatments were subjected to OGD/R as described in the Methods section (white bar graphs represent normal conditions, black bar graphs represent the OGD/R exposed cells). Treatments with PF-431396 and L-NAME were 5μM for 45 minutes and 100μM for 40 minutes prior to the MTT assay; NT: non-transfected. n=4-8, *p<0.05 vs vehicle-control cultures , *p<0.05 vs NT-control cultures , #p<0.05 vs vehicle OGD/R , #p<0.05 vs NT OGD/R , $ p<0.05 vs PYK2 siRNA+H_2O_2 . PYK2 was inhibited with PF-431396 (5μM for 45 minutes) or was silenced for 48hr.

In a separate series of experiments, inhibition with PF-431396 or silencing of PYK 2 of H9c2 resulted in increased cGMP, in line with the low level phosphorylation of eNOS on Y656 in H9c2 under baseline conditions (Fig. 10A,10B).

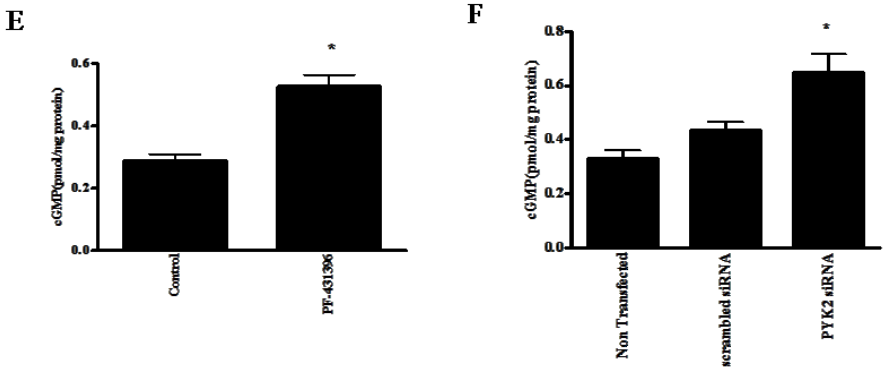
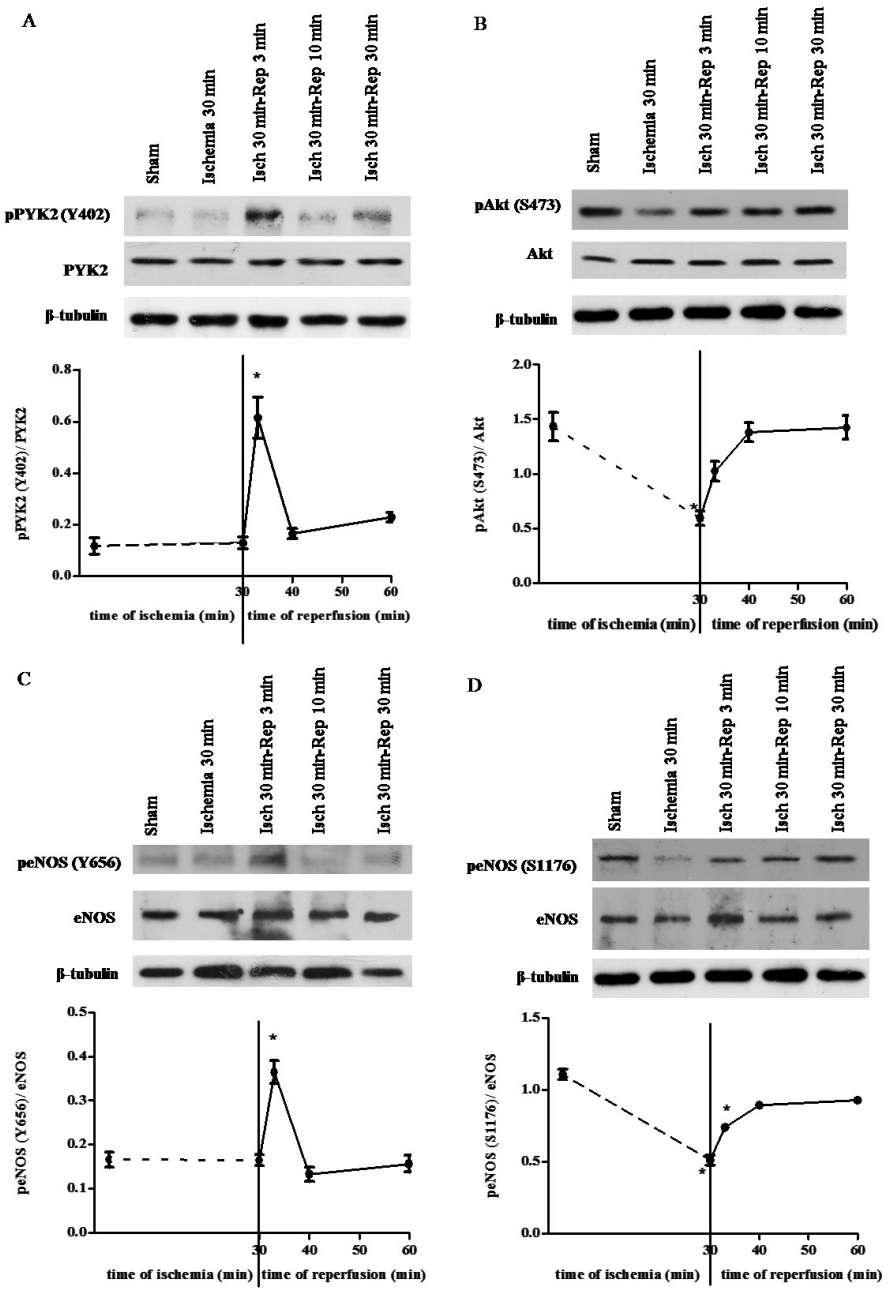


Figure 10: Inhibition of PYK 2 increases cGMP levels. Cellular cGMP was extracted and measured using a commercially available enzyme immunoassay (EIA) kit (Assay Designs;

Ann Arbor, MI). PF-431396 pretreatment: 5μM for 45 minutes; n=4 per group; *p<0.05 vs Control(A),*p<0.05 vs Non Transfected(B)

1.4.5. Time-dependent phosphorylation of PYK2 and eNOS in I/R

To evaluate the relevance of our findings *in vivo*, changes in PYK2 and eNOS phosphorylation were monitored at different time points of ischemia/reperfusion following LAD ligation. In these experiments, we observed increased phosphorylation of PYK2 in the early minutes of reperfusion (Fig. 11A). Phosphorylation of eNOS Y656, followed the same phosphorylation pattern with Y656 peaking at the 3rd minute of reperfusion (Fig. 11C). Akt and S1176 eNOS phosphorylation were reduced at the end of ischemia; phosphorylation started to increase during early reperfusion and showed an increasing trend throughout the time period tested (Fig. 11B, 11D). In contrast, Thr495 phosphorylation remained unchanged throughout the time course studied (Fig. 11E). To evaluate the amount of NO produced at the end of ischemia and during reperfusion, cGMP was measured as a surrogate marker of biologically active NO. cGMP levels were reduced in the end of the ischemic insult. cGMP levels reached a trough at the 3rd minute of reperfusion, when the highest levels of eNOS Y656 phosphorylation were observed (Fig. 11F); cGMP displayed an upward trend after that paralleling the Y656 de-phosphorylation and S1176 phosphorylation.


A
Sham
Ischemia 30 min
Isch 30 min-Rep 3 min
Isch 30 min-Rep 10 min
Isch 30 min-Rep 30 min
pPYK2 (Y402)
PYK2
β-tubulin
pPYK2 (Y402)/ PYK2
time of ischemia (min)
time of reperfusion (min)
B
pAkt (S473)
Akt
β-tubulin
pAkt (S473)/ Akt
time of ischemia (min)
time of reperfusion (min)
C
peNOS (Y656)
eNOS
β-tubulin
peNOS (Y656)/ eNOS
time of ischemia (min)
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time of ischemia (min)
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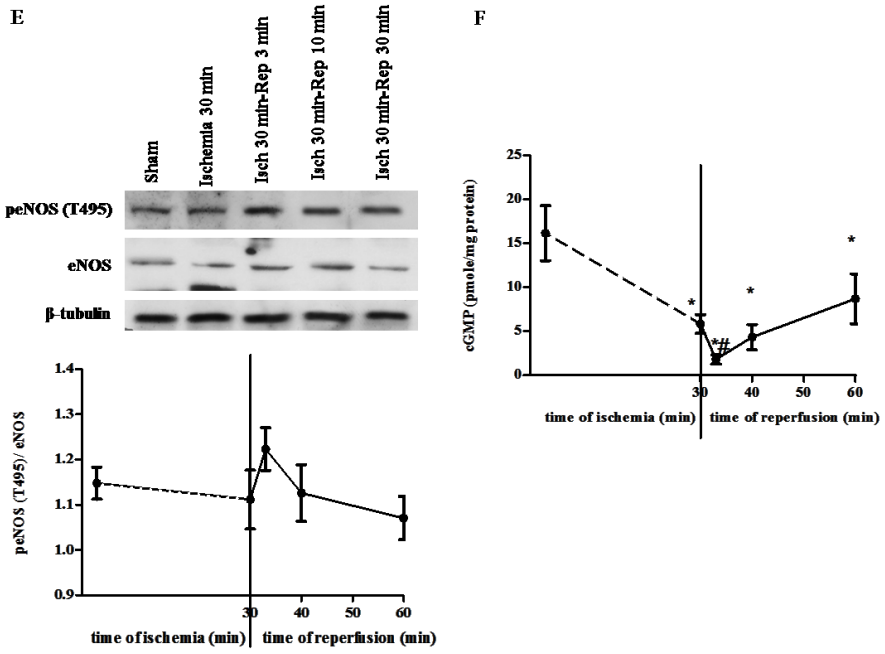
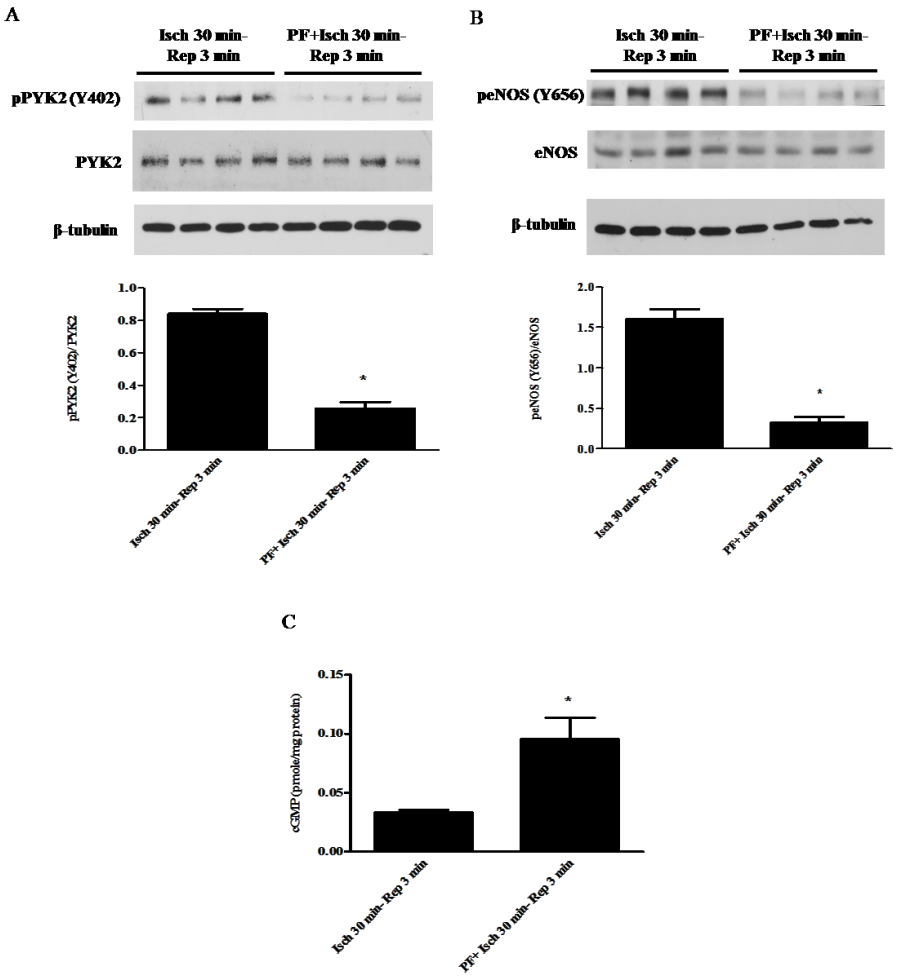


Figure 11: Activation of the PYK2-eNOS axis during ischemia/reperfusion injury *in vivo*. Mice were subjected to ischemia for 30min, or ischemia and reperfusion for 3, 10 or 30min. Tissues were collected and PYK2 (A), Akt (B), eNOS phosphorylation (C-E) and cGMP levels (F) were determined. A-E Representative western blots and densidometric analysis of (A) phospho-PYK2(Y402); (B) phospho-Akt (S473); (C) phospho-NOS (Y656); (D) phospho-eNOS (S1176), (E) phospho-eNOS (T495). Results were normalized for total protein levels. β tubulin is presented as reference protein. (F) cGMP levels at different times post-ischemia and reperfusion. n=5 per group; *p<0.05 vs sham, #p<0.05 vs ischemia 30min (F)

1.4.6. Inhibition of PYK2 enhances eNOS signaling and reduces infarct size following ischemia/reperfusion

Administration of the PYK2 inhibitor in mice subjected to myocardial infarction, resulted in reduced PYK2 autophosphorylation (Fig. 12A), which in turn lead to attenuated phosphorylation of eNOS on Y656 (Fig. 12B) in the early phase of reperfusion. In line with this observation, cGMP levels increased at the 3rd minute of reperfusion in the presence of a PYK2 inhibitor (Fig. 12C).



<u>Figure 12:</u> Inhibition of PYK2 blocks Y656 eNOS phosphorylation and restores cGMP levels during early reperfusion *in vivo*. Mice were subjected to ischemia (30min) and reperfusion for 3min. One group of mice received PF-431396 as iv bolus at 5μg/g 10 minutes before myocardial ischemia. Tissues were then collected and PYK2 (A), and eNOS phosphorylation (B) were determined. Representative western blots and densidometric analysis of A. phospho-PYK2(Y402); B. phospho-NOS (Y656). Results were normalized for total protein levels. β-tubulin is presented as reference protein. (C) cGMP levels in the ischemic myocardium in mice receiving vehicle or PF-31396 treatment, n=6 per group; *$p<0.05$ vs no PF-31396.

To test whether PYK2 inhibition impacts on infarct size, mice were treated with PF-431396 either prior to the onset of ischemia (pre-treatment) or the end of ischemia (post-treatment). PF-431396 did not alter heart beat or mean arterial blood pressure (data not shown). Pre-treatment or post-treatment with PF-431396 resulted in a significant reduction of the myocardial infarct size vs the control group (10.1%±1.3%, 8.1%±2.4% respectively vs 45.4%±3.9%) (Fig. 13A & 13C). The area at risk was similar among groups (Figure 13B). The beneficial effect of the PYK2 inhibitor was absent in eNOS KO mice, suggesting that PF-431396-induced protection was eNOS-dependent (Figures 13A & 13C). Areas at risk were not different among the treatments in eNOS KO mice (Fig.13B)

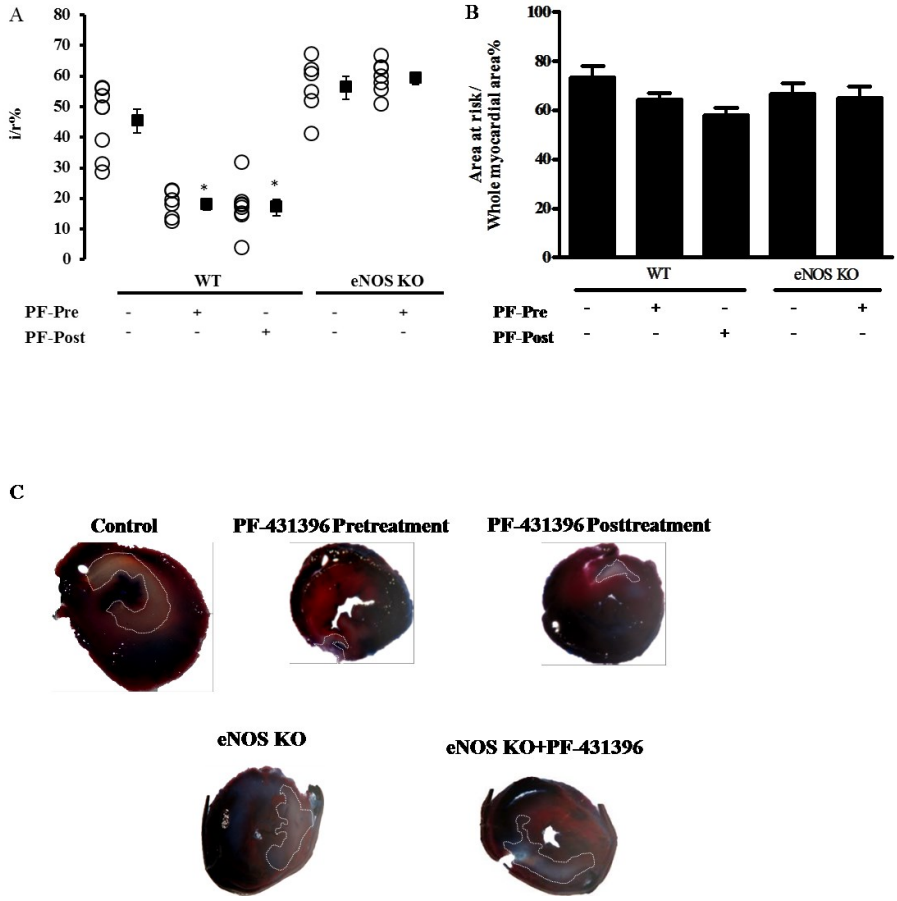


Figure 13: Inhibition of PYK2 is cardioprotective in wild-type, but not eNOS KO mice. Mice were subjected to LAD ligation and infracted area, area at risk and total area were determined. A. Infarcted to area at risk ratio as % (% i/r). n=8 for control group, n=9 for PF-431396 pre-treatment group, n=7 for PF-431396 post-treatment group; n=6 for eNOS KO group and n=7 for PF-431396 pretreatment in eNOS KO group; *p<0.05 vs wt control. B. Ratio of area at risk to whole myocardial area. p=NS among groups. C. photomicrographs of representative pictures from different treatment groups.

1.5. Discussion

The main findings of the present study are: 1) PYK2 is activated in response to oxidative stress in cardiomyocytes *in vitro* and during ischemia-reperfusion *in vivo*, 2) once activated, PYK2 promotes dual phosphorylation of eNOS on S1177 and Y656, 3) Y656 phosphorlyation has a dominant, inhibitory effect on eNOS activity, 4) activation of PYK2 results in decreased cardiomyocyte viability *in vitro* and *in vivo*, 4) PYK2 inhibition alleviates the inhibitory effect on eNOS, limiting infarct size.

PYK2 is a non-receptor tyrosine kinase that can be activated by calcium [303] and stress signals [304]. PYK2 can be viewed as a redox-sensitive kinase, since its activation occurs following exposure to oxidative stress or in response to agents that promote ROS production, like angiotensin II and endothelin-1 [305-309]. PYK2 activation in the heart is linked to cardiac remodeling [310, 311], hypertrophic responses [312-314] and dilated cardiomyopathy [315].

In our *in vitro* experiments, we observed that short-term treatment of cardiomyocytes with H_2O_2 activates PYK2 kinase. This observation is in line with the finding that H_2O_2 promotes PYK2 phosphorylation in endothelial cells[307] and that NADPH oxidase activation results in PYK2 phosphorylation in H9c2 cells[316]. Since PYK2 has been shown to promote eNOS phosphorylation both directly on Y656[317] and indirectly on S1176 through PI3K/Akt[318, 319], we examined the ability of H_2O_2 to trigger eNOS phosphorylation on these residues in cultured cardiomyocytes. Indeed, we found that exposure to H_2O_2 increased phosphorylation of eNOS on both S1176 and Y656. In line with our observations, Sartoretto el al reported that H_2O_2 increased phosphorylation on S1176 of eNOS in H9c2 in a Ca^{2+}-dependent manner [320]. In subsequent experiments we found that PYK2 inhibition abolished phosphorylation on both sites, suggesting that H_2O_2-induced eNOS phosphorylation is PYK2-mediated. Although the observed phosphorlyation of Y656 by PYK2 can be easily explained by direct phosphorylation, the modification of serine would require an intermediate ser/thr kinase. To test the mechanism of PYK2-induced S1177 phosphorlyation, we evaluated the ability of PYK2 to activate PI3k/Akt pathway, the prominent kinase that phosphorylates eNOS on this serine residue. We observed that upon H_2O_2 exposure, the p85 catalytic subunit of PI-3K, an upstream activator of Akt, was recruited to PYK2 and phosphorylated. In addition, H_2O_2-induced Akt phosphorylation was inhibited by PYK2 pharmacological inhibition or PKY2 gene knockdown, confirming that Akt is downstream of PYK2. Our findings are in agreement with previous reports that Akt is a downstream effector of PYK2 in neonatal human and rat ventricular cardiomyocytes[318, 321].

As described above in cardiomyocytes we observed a simultaneous dual effect on eNOS phosphorylation state, not observed in other cell types[322]. Other studies have reported that H_2O_2 exert a concentration dependent but not simultaneous dual effect on eNOS phosphorylation which is also reflected by changes in eNOS activity[322]. At low concentrations (e.g., 30 μM), H_2O_2 inhibited basal eNOS activity, whereas at high concentrations (300–500 μM) H_2O_2 stimulates the influx of Ca^{2+} into endothelial cells[323], and NO production[322]. The latter phenomenon has also been reported by other authors[324, 325]. We recently reported that the phosphorylation of Y657 by PYK2 abolishes eNOS activity; mutation of Y657 to glutamate or aspartate eliminates NO generation [317]. However, the effect of Y657 phosphorylation on eNOS activity when S1177 is also phosphorylated had not been examined. We, thus, transfected HEK293 cells with the Y657D/S1177D phosphomimetic mutant and compared its activity with that of wild-type and Y657D eNOS. We observed eNOS activity of the Y657D/S1177D eNOS to be severely impaired, similar to that of Y657

eNOS. Thus, phosphorylation of Y657 exhibits a dominant effect over S1177, blocking eNOS activity irrespectively of the phosphorylation status of the serine residue. Our findings, might also explain why some agents that increase S1177 phosphorylation fail to increase NO production. The concentration-time dependent effect of H_2O_2 on eNOS regulation may solve the discrepancy of our findings to the reported increased eNOS activity in cardiomyocytes under H_2O_2 treatment[326]. Taken together, these results demonstrate that the dominant determiner of eNOS activity is the tyrosine residue located in the FMN binding domain of the enzyme.

To correlate the biochemical observations to functional responses, we performed cell viability assays in cells exposed to H_2O_2, assessing either complex II activity by determining MTT conversion, or cell membrane integrity by measuring LDH release. We observed that inhibition or silencing of PYK2 kinase rendered the cardiomyocytes less susceptible to injury in response to H_2O_2 and oxygen/glucose deprivation. In line with our finding, chronic PYK2 activation was correlated to increased cardiomyocyte death in heart failure [310]. Interestingly, the beneficial effect of PYK2 blockade was prevented by eNOS inhibition. Published reports have so far studied PYK2 and NO as independent variables in cardiomyocyte survival. This series of experiments allowed us to link the two pathways and formulate the hypothesis that PYK2 activation during oxidative stress injury inactivates eNOS, minimizing NO bioavailability, thus, leading to increase cell death. Inhibition of PYK2 restores eNOS functionality, exerting a protective effect in response to noxious stimuli.

To test the relevance of our *in vitro* findings to the injury that follows I/R in vivo, we used a mouse model of LAD ligation. In these experiments we observed that PYK2 phosphorylation (and presumably activity) is low at the end of ischemia; early during reperfusion PYK2 activity shows a peak and phospho-PYK2 levels return to baseline levels soon after that. The kinetics of PYK2 activation are reminiscent of the kinetics of ROS generation that are formed in high amounts early in reperfusion[327, 328], making it tempting to speculate that ROS is indeed the trigger for enhanced PYK2 activity. In line with the fact that eNOS is a substrate for PYK2, Y656 showed an identical time course of phosphorylation demonstrating higher levels 3min after the onset of reperfusion. In contrast, Akt and S1177 phosphorylation are lowest at the end of the ischemic period and increase with after re-establishing blood flow. A similar time course of Akt and eNOS S1177 phosphorylation has been reported by Inserte et al. who observed low eNOS S1177 phosphorylation at the end of ischemia that gradually increased towards normoxic levels in isolated perfused hearts[329]. Others have also reported low Akt phosphorylation at the end of ischemia[98]. The low level of S1177 phosphorlyation we observed at the onset of reperfusion correlates well with the finding that eNOS activity declines throughout ischemia[330] and is further supported by finding by Cai et al.[331] who showed a time-dependent decrease on S1177 phosphorylation with ischemia. On the other hand, T495 phosphorylation similarly to what was observed *in vitro* remains unaltered, suggesting that this residue does not contribute to changes in eNOS activity during I/R. No changes on T495 during I/R have also reported by other investigators [332].

To assess the effect of altered phosphorylation eNOS status on eNOS activity, we measured cGMP, a surrogate marker of biologically active NO production. cGMP levels were low at the end of ischemia and dropped even further at the 3rd minute of reperfusion, paralleling the increase in phosphorylation in the dominant eNOS Y656 site. Thereafter, cGMP levels began to rise reflecting the drop in Y656 and the increase in S1177 phosphorylation. It should be noted that the levels of cGMP during reperfusion failed to reach those measured in sham

animals, in spite of restoration of favorable eNOS phosphorylation pattern. NO production from eNOS during I/R in known to be limited by BH_4 availability, eNOS coupling and dimerization [329, 330] and intracellular acidification [333]. These factors, along with the ongoing ROS production contribute to lower levels of biologically active NO preventing restoration of cGMP levels. In agreement with our findings, cGMP has been shown to be reduced after I/R [334-336].To prove causality between enhanced Y656 phosphorylation and low NO output *in vivo*, we determined cGMP levels at the 3rd minute of reperfusion in the presence and absence of a PYK2 inhibitor PF-431396. Pharmacological inhibition of PYK2 inhibited eNOS Y656 phosphorylation and enhanced cGMP accumulation, providing evidence that NO output is augmented and eNOS activity is de-repressed, once PYK2 is blocked.

To assess the functional implications of alterations in the pattern of eNOS phosphorylation, we measured myocardial infarct size *in vivo*. In agreement to our biochemical and *in vitro* observations, we found that pharmacological inhibition of PYK2 kinase throughout ischemia or at the onset of reperfusion that prevents the decline in eNOS activity in the critical initial minutes of reperfusion, reduced myocardial infarct size. To prove that the action of the PYK2 inhibitor PF-431396 is eNOS-dependent, we repeated the experiments is eNOS KO mice. Interestingly, in spite of the importance of eNOS in cardioprotection, similarly to what has been shown by other investigators[98, 337] we observed that infarct size in eNOS KO mice was comparable to that of wild-type controls. More importantly, the cardioprotective effect of PYK2 inhibition was abolished in mice lacking eNOS, lending further credence to our hypothesis that tyrosine phosphorylation of eNOS in Y656 is a major determinant of infarct size.

In summary, our *in vitro* and *in vivo* findings taken together demonstrate a negative role for PYK2 I/R injury and myocardial infarction and underscore the importance of eNOS in myocardial infarction. PYK2 activation results in dual eNOS phosphorylation: a direct phosphorylation on eNOS Y656 and an indirect, Akt-mediated phosphorylation on Ser1176. The functional consequence of these post-translational modifications is a reduction of NO output in the early minutes of reperfusion, which results in myocardial cell death. Thus, PYK2 emerges as a novel therapeutic target that restores eNOS signaling and function in I/R injury and minimizes cardiac necrosis.

1.6. Study limitations

eNOS activity and/or NO levels are harder to assess in *in vivo* studies. Although cGMP levels are a good measure of biologically active NO, they are not synonymous to eNOS activity, due to their dependence on (soluble and particulate) guanylate cyclase and phosphodiesterase activity, among others. Readers are encouraged to consider this, while making their own analysis of the data presented herein. In addition, PF-431396 is a more potent inhibitor of FAK (IC_{50}=2nM) vs PYK 2 (IC_{50}=11nM) and protective roles for FAK have been reported in myocytes[338]. Use of any pharmacological inhibitor raises questions regarding possible off-target effects. However, the fact that we obtained similar results after siRNA knockdown of PYK2 argues in favor of a role for PYK2 in the cardioprotective responses. Further validation of PYK2, as a potential target for cardioprotection is warranted in a cardiomyocyte-specific PYK2 knockout model. In addition, it is unclear form our experiments whether the target of PYK2 is in the endothelial cells or the cardiomyocytes themselves. However, as it has been reported the endothelial derived NO during ischemia reperfusion injury plays a minor role compared to the cardiomyocyte derived NO [339]. The finding that PYK2 activation drives the

angiogenic response by enhancing Akt and eNOS activity (through eNOS phosphorylation on S1176)[319] seems to be at contrast to the present study. Transient inhibition of eNOS activity by PYK2 direct phosphorylation on Y656 will have less of an impact on angiogenesis (a process that requires days to occur); on the other hand, transient inhibition of NO during the initial seconds to minutes of re-establishing blood flow, will have long-term detrimental effects on cardiac function after reperfusion. Most importantly, as with the many other experimental treatments that have failed during translation, observations herein were made in otherwise healthy animals. The effectiveness of PYK2 inhibition would have to be proven in the face of co-morbidities and/or after co-administration of other commonly prescribed medications.

Experimental Study II:

Paradoxical requirement of eNOS in nitroglycerin-induced cardioprotection.

2.1. Abstract

Aim: Nitroglycerin (NTG) remains a first-line treatment for pain relief during AMI. It acts as a vasodilator and reduces left ventricular preload volume, wall tension, and therefore, myocardial oxygen demands. However, the decrease in arterial pressure would, result in a reduction in coronary perfusion pressure and thus a potential increase in infarct size. In addition, prolonged administration of NTG results in nitrate tolerance, limiting its clinical use. Herein, we sought to determine whether acute low dose NTG exerts direct effects on myocardial infarct size as a postconditioning mimetic without alterations in hemodynamics or induction of nitrate tolerance in an experimental model of ischemia-reperfusion injury.

Methods and Results**:** Low dose IV NTG reduced infarct size similar to PostC group in anesthetized male rabbits without affecting haemodynamics. Co-administration of PI3K or NOS inhibitors along with NTG abrogated NTG's beneficial effect. Inhibition of iNOS, did not affect the cardioprotection afforded by NTG whilst inhibition of nNOS increased NTG's benefit. To further evaluate the role of eNOS in NTG induced cardioprotection, we treated eNOS(-/-) mice with NTG; NTG had no effect on infarct size in eNOS(-/-) mice, although its cardioprotective actions were reproduced in the murine wild type heart but not in the wild type ones with nitrate tolerance. In addition, eNOS and AKT phosphorylation were higher in NTG-treated rabbits compared to the control group. However, no phosphorylation of eNOS was detected in the murine hearts. Mechanistically, NTG protected through a CypD-dependent manner as NTG failed to confer additional protection in hearts from CypD(-/-) mice, which already exhibited smaller infarct size than their wild type littermates. In addition, NTG offered cardioprotection in ApoE (-/-) mice indicating that it could be a potent cardioprotective agent in the presence of endothelial dysfunction conditions. Interestingly, NTG effects were mediated due to an eNOS mediated nitro-oxidative stress reduction in the ischemic myocardium.

Conclusion**:** Low dose IV NTG induces pharmacological postconditioning and reduces myocardial infarct size. Its effects are mediated through activation of the PI3K-AKT pathway and inhibition of mPTP. The observed crosslink between eNOS and reduced nitro-oxidative stress is a novel mechanism of NTG infarct limiting effects. Further studies are mandatory for the evaluation of eNOS activation upon NTG treatment.

2.2. Aim

Glyceryl-trinitrate (commonly termed nitroglycerin), is a potent nitro-vasodilator, which has been shown to increase NO species under clinical conditions[238, 340]. The positive effects of NTG arise from its ability to promote vasodilation, resulting in increased blood flow to the heart[341]. However, while low dose NTG effects are evident only in systemic veins where the venodilator effect reduces cardiac preload and further decreases myocardial wall stress[239], thus restoring the equilibrium of oxygen and nutrients supply-demand in the ischemic heart, higher doses of NTG balance venous and arterial dilating effect, affecting myocardial perfusion and thus potentially increases infarct size[342]. In addition, sustained NTG administration causes tolerance and is associated with pro-oxidant effects, endothelial dysfunction and increased sensitivity to vasoconstrictors[240, 241, 343] furthermore it causes tolerance to ischemic preconditioning effects[242, 243]. Its beneficial effects given before the ischemic insult on the limitation of myocardial infarction have been described[344]; Although, it has been shown that the protective effect of ischemic preconditioning can be mimicked pharmacologically with nitric oxide (NO) donors[244], the effect of NO donors as pharmacological postconditioning agents have only superficially been assessed.

Thus, the purpose of the present study is

1. To evaluate the possible cardioprotective effects of low dose IV nitroglycerin, administered at the end of ischemia and reperfusion.
2. To evaluate the involvement of PI3K/Akt-nitric oxide synthases pathway on nitroglycerins' possible infarct size limiting effects.
3. To assess the role of NTG in the mPTP opening and oxidative stress levels in the ischemic heart.
4. To evaluate possible cardioprotective actions of NTG in a model of endothelial dysfunction.

2.3. Materials and Methods

2.3.1. Animals

Animals received proper care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health. Animals received the usual laboratory diet and all studies were approved by the animal research ethics committees in Athens and Mainz.

In the present study we used 117 white male New Zealand rabbits 2.7-3.1 kg in total, 34 male mice C57BL/6 as wild type 12-16 weeks old, 12 male eNOS(-/-) mice 13-16 weeks old, 12 male CypD(-/-) mice 12-14 weeks old and 14 male ApoE(-/-) mice 12-14 weeks old.

2.3.2. Surgical Procedures

2.3.2.1. Rabbit in vivo model of ischemia/reperfusion injury

Rabbits were anesthetized with sodium thiopentone at a dose of $30mg \cdot Kg^{-1}$ by bolus injection into a peripheral vein. Animals were placed in a supine position and ECG recording was induced by use of specific electrodes (PowerLab 4.0, ADInstrumnets, Campbridge, UK). Recording was achieved through the appropriate software (Labchart 7.0, ADInstruments, Cambridge, UK). Depth of anesthesia was evaluated by animals reflex and ECG recording. Anesthesia follows tracheotomy for proper intubation with a respirator for small animals (MD Industries, Mobile, AL, USA) for mechanical ventilation at a rate adjusted to maintain normal blood gases. Carotid was revealed and catheterized with a 20G catheter connected to a fluid filled transducer for invasive blood pressure recording. Invasive open of the chest with a left thoracotomy exposed the beating heart. The pericardium was opened and a 3-0 silk suture was passed around the left anterior descending artery. Ischemia was induced by pulling the thread through a small piece of soft tubing, which was firmly positioned against the coronary arterial wall with the aid of a small clamp. Ischemia resulted in ST elevation on the ECG and a discolor of the ischemic myocardium. At the end of ischemic period the snare was opened, the artery refilled and the myocardium reperfused. After the end of reperfusion, hearts were excised, mounted on an apparatus and perfused with normal saline for 2 minutes for blood removal. Then the coronary ligature was retightened at the same site and 10 ml of green fluorescent microspheres (Duke Scientific Corp. Palo Alto CA) were infused for the separation of the non ischemic area from the area at risk. Hearts were kept in the refrigerator for 24 hours and cut into 3 mm thick sections. The slices were stained with triphenyltetrazolium chloride (TTC) at 37^0C and immersed in 10% formaldehyde overnight. With a wavelength of 366 nm UV light, we separated the area at risk from the infracted zone of the heart and we traced all the areas onto an acetate sheet in order to estimate the volumes of these areas in cm^3. The infarct to risk area ratio (%I/R) was calculated. The infracted, the risk and the normal areas were traced onto an acetate sheet, which had been placed over the top glass plate. The tracings were subsequently scanned with the Adobe Photoshop 6.0 and measured with the Scion Image program. The areas of myocardial tissue at risk and infracted were automatically transformed into volumes. Infarct and risk area volumes were expressed in cm^3 and the percent of infarct to risk area ratio (%I/R) was calculated[248].

2.3.2.1.1. Murine in Vivo Model of Ischemia-Reperfusion Injury

C57BL/6 wild type, eNOS(-/-), CypD(-/-) and ApoE(-/-) male mice 12-16 weeks old were subjected to myocardial ischemia reperfusion injury.

Methods described in Experimental Study I section 1.3.12.1. [345].

2.3.2.2. Experimental protocol

2.3.2.2.1. Experimental protocol in the rabbit cohort

75 New Zealand white male rabbits weighted 2.7-3.1 kg were subjected to 30 minutes regional ischemia of the myocardium, followed by 3 hours of reperfusion and were randomized into 12 groups as follows (Study protocol is presented on Figures 1A, 2A, 4A) .

Pharmacological inhibitors were administrated in our in vivo models as previously described by our group or others that are effective in the rabbit ischemia reperfusion model (references included in each administration protocol):

Control group (n=8): No additional intervention

PostC group (n=7): Application of 8 cycles of 30 sec ischemia reperfusion immediately after sustained ischemia.

NTG group (n=7): Administration of NTG IV at a dose of 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for a total time of 65 min starting 10 min prior to reperfusion[244, 246].

NTG+W group (n=7): Administration of NTG as in NTG group and the inhibitor of the PI3K inhibitor (Wortmannin) IV bolus at the 19th min of ischemia at a dose of 60 $\mu g \cdot kg^{-1}$ as previously described[346].

NTG+L-NAME (n=7): Administration of NTG as in NTG group and the inhibitor of the synthase of NO (NOS) (L-NAME) IV bolus at the 19th min of ischemia at a dose of 10 $mg \cdot kg^{-1}$ as previously described[248].

NTG+7-NI group (n=7): Administration of NTG as in NTG group and the inhibitor of the neuronal synthase of NO (nNOS) (7-Nitroindazole) IV bolus 20 min prior to ischemia at a dose of 50 $mg \cdot kg^{-1}$ as previously described[347].

NTG+1400W group (n=6): Administration of NTG as in NTG group and the inhibitor of the inducible synthase of NO (iNOS) (1400W) IV bolus 30 min prior to ischemia at a dose of 10 $mg \cdot kg^{-1}$ as previously described[347].

NTG+DT2 group (n=7): Administration of NTG as in NTG group and the inhibitor of PKG-I (DT2) IV bolus 10 min prior to ischemia at a dose of 0.25 $mg \cdot kg^{-1}$ as previously described[345].

W (n=6): Administration of the inhibitor of the PI3K inhibitor (Wortmannin) IV bolus at the 19th min of ischemia at a dose of 60 $\mu g \cdot kg^{-1}$ as previously described[346].

L-NAME (n=7): Administration of the inhibitor of the synthase of NO (NOS) (L-NAME) IV bolus at the 19th min of ischemia at a dose of 10 $mg \cdot kg^{-1}$ as previously described[248].

7-NI (n=6): Administration of the inhibitor of the neuronal synthase of NO (nNOS) (7-Nitroindazole) IV bolus 20 min prior to ischemia at a dose of 50 $mg \cdot kg^{-1}$ as previously described[347].

1400W group (n=6): Administration of the inhibitor of the inducible synthase of NO (iNOS) (1400W) IV bolus 30 min prior to ischemia at a dose of 10 $mg \cdot kg^{-1}$ as previously described[347].

In a second series of experiments twenty five rabbits (5 per group of Control, PostC, NTG, NTG+W and NTG+L-NAME groups) were subjected to the same interventions up to the 10th minute of reperfusion, where tissue samples from the ischemic area of myocardium were collected, emerged in liquid nitrogen and stored at -80°C for western blot analysis of Akt and eNOS assessment.

Additionally, in a third series of experiments, sixteen rabbits (4 per group of Control, PostC, NTG and NTG+L-NAME groups) were subjected to the same interventions up to the 10th minute of reperfusion, where tissue samples from the ischemic area of myocardium were collected, embedded in OTC compound (Tissue Teck) and emerged in liquid nitrogen and stored at -80°C for ROS evaluation by DHE staining.

2.3.2.2.2. Experimental protocol in wild type C57BL/6 mice

42 wild type C57BL/6 male mice 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions. (Study protocol is presented on Figure 3A).

Control group (n=9): Vehicle NaCl 0.9% administered 3 times per day for 3,5 days before the I/R induction. Mice were also treated with NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml . h-1 .

NTG group (n=8): Vehicle NaCl 0.9% administered 3 times per day for 3,5 days before the I/R induction. Administration of NTG at a dose of 24μg . kg-1 . min-1 in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 $ml \cdot h^{-1}$ [348].

NTG tolerance group (n=7): NTG in NaCl 0.9% at a dose of 20 mg/kg administered 3 times per day for 3,5 days to induce nitrate tolerance25 before the I/R induction. Mice were also treated with NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml . h-1 .

The above groups of animals were used for infarct size determination.

Eighteen additional animals (6 per group) were subjected to the above intervention up to the 10th minute of reperfusion in which tissue samples from the ischemic region of the heart were embedded in OTC Compound (Tissue Teck) for ROS evaluation by DHE staining.

2.3.2.2.3. Experimental protocol in eNOS(-/-) mice

Twelve eNOS(-/-) male mice 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions. (Study protocol is presented on figure 6A)

eNOS(-/-) group (n=6): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 $ml \cdot h^{-1}$

eNOS(-/-)+NTG group (n=6): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}

2.3.2.2.4. Experimental protocol in CypD(-/-) mice

Fourteen CypD(-/-) male mice 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions.

CypD(-/-) group (n=6): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}

CypD(-/-)+NTG group (n=6): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}

2.3.2.2.5. Experimental protocol in ApoE(-/-) mice

Fourteen ApoE(-/-) male mice 13-16 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions.

ApoE(-/-) group (n=7): Vehicle NaCl 0.9% administered at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}

ApoE(-/-) +NTG group (n=7): Administration of NTG at a dose of 24μg · kg^{-1} · min^{-1} in NaCl 0.9% at the 20th minute of ischemia for a total time of 65 min, with a steady rate of infusion of 0.2 ml · h^{-1}

2.3.3. Western Blot Analysis

Described in Experimental Study I section 1.3.4.

In the present study we used as primary antibodies phospho eNOS(Ser1177), eNOS, phospho Akt(Ser243), Akt, beta tubulin (Cell signaling Technologies).

2.3.4. DHE staining of cardiac RONS formation

Cardiac ROS production was qualitatively detected by DHE (1 μM)-derived fluorescence in heart tissue sections as described previously for vascular ROS formation[349].

2.3.5. Statistical analysis

All results are presented as mean ± standard error mean. Comparisons of numeric

variables among the groups were analyzed using One-way Analysis of Variance model (ANOVA) with Bonferroni correction and with Tukey post-hoc analysis. A calculated p value of less than 0.05 was considered to be statistically significant.

2.4. Results

2.4.1. Hemodynamic parameters

Characteristics and hemodynamic variables for study groups are presented in Table 1.for rabbit groups and Table 2.for mice groups. Nitroglycerin administered in the described dose did not change the hemodynamic profile of the treated animals. No significant differences were observed between the groups.

Table 1: Haemodynamic variables of the rabbit in vivo ischemia reperfusion model

Study group	HW (g)	Baseline		20 min Ischemia Drug infusion				180 min Reperfusion 1st minute		180th minute	
		HR	MAP	HR	MAP	HR	MAP	HR	MAP	HR	MAP
Control	7.4±0.2	273±11	84±6	270±13	82±5	270±11	82±4	268±12	81±6	266±11	77±5
PostC	7.1±0.3	279±12	81±5	274±12	79±4	275±10	79±5	273±13	76±5	269±10	72±6
NTG	7.5±0.5	276±13	80±4	271±11	77±5	271±12	77±3	270±12	75±4	268±12	76±4
NTG+LNAME	7.6±0.3	275±10	83±4	270±9	80±3	270±11	79±5	268±9	80±3	265±11	78±6
NTG+7NI	7.4±0.5	274±13	82±5	268±12	79±7	269±9	78±4	269±12	77±7	263±9	75±4
NTG+Wortmannin	7.2±0.6	275±11	84±7	269±10	81±6	269±13	81±3	267±10	78±6	265±13	78±3
NTG+SPT	7.7±0.4	277±14	82±6	272±14	78±5	272±12	79±3	269±13	76±4	267±12	74±4
NTG+DT2	7.5±0.3	273±15	87±5	267±11	83±4	266±14	83±5	265±10	78±5	264±14	78±5
LNAME	7.1±0.6	274±11	81±6	269±10	78±5	270±12	78±6	270±11	77±5	266±12	73±6
7NI	7.4±0.5	273±15	79±5	270±14	76±6	269±13	77±5	270±10	74±6	267±13	72±5
Wortmannin	7.2±0.6	272±12	78±7	266±11	75±4	267±12	75±7	265±12	74±4	262±12	72±4
SPT	7.5±0.3	275±9	83±3	270±12	79±3	270±9	78±4	269±10	77±7	268±9	75±3

HW: Heart weight in g, HR: Mean heart rate in beats/min, MAP: Mean arterial blood pressure in mmHg.

Table 2: Haemodynamic variables of the murine in vivo ischemia reperfusion model

HW	Baseline		20 min Ischemia Drug infusion				180 min Reperfusion 1st minute		180th minute	
	HR	MAP	HR	MAP	HR	MAP	HR	MAP	HR	MAP
152±5	384±3	97.1±1.9	378±2	95.2±1.9	377±2	96.0±1.6	373±2	99.6±1.3	370±2	93.4±1.2
151±5	390±4	98.6±4.8	385±4	99.8±2.9	385±4	99.8±3.0	380±3	100.6±3.3	374±2	105.8±0.5

HW: Heart weight in mg, HR: Mean heart rate in beats/min,
MAP: Mean arterial pressure in mmHg.

2.4.2. Nitroglycerin induces cardioprotection through PI3K activation in rabbits.

In rabbits under ischemia/ reperfusion no significant differences were detected in the areas at risk among groups (Figure 1B). PostC reduced infarct size compared to the control group (23.3±0.99 vs 47.7±1.8%, p<0.05). Administration of NTG at the end of ischemia protected the ischemic myocardium to a similar extent as PostC (23.06±2.7 %, p=NS *vs* PostC) (Figure 1C). Co-administration of wortmaninn along with NTG did not affect area at risk (Figure 1C) but abrogated the infarct limiting effects of NTG (40.66±2.4%, *vs* 23.06±2.7 %, p<0.05) (Figure 1C). NTG administration increased the phosphorylation of Akt at ser478 at the ischemic myocardium during reperfusion whereas it was reduced after administration of wortmannin (Figure 1D).

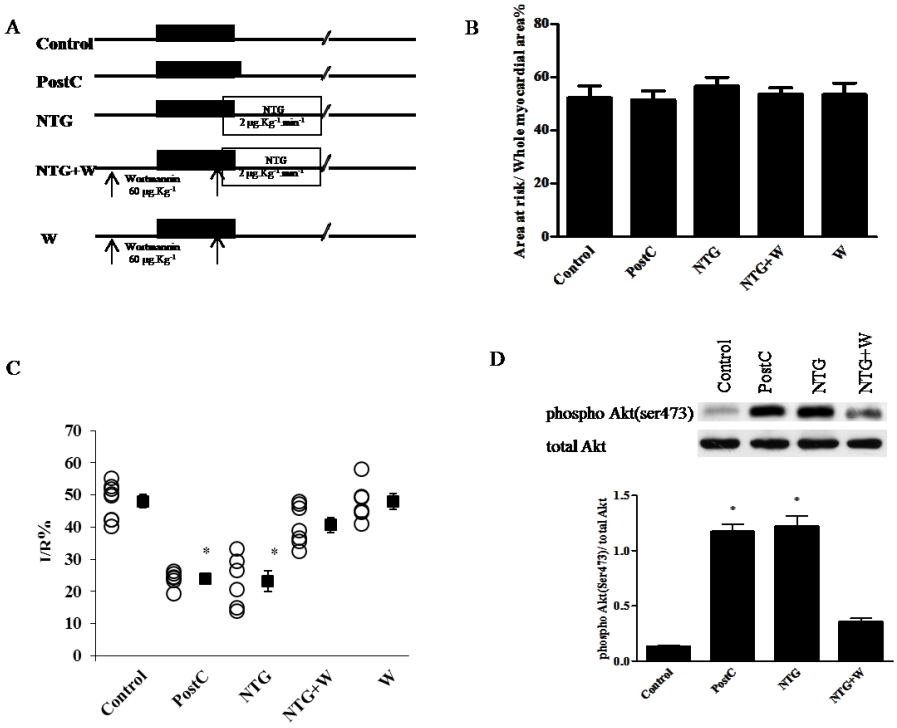


Figure 1. **NTG reduces myocardial infarct size and activates Akt.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative blots for pAkt/tAkt are shown along with the densitometric analysis from the total number of animals per group (n=5). (*p<0,05 versus Control group)

NOS inhibition did not alter area at risk among groups (Figure 2B). L-NAME reversed the cardioprotective effects of NTG (39.3±1.4% *vs* 23.06±2.7 %, p<0.05) (Figure 2C). Selective inhibition of nNOS increased NTG benefit (12.15±0.74%, p<0.05) whilst 7-NI itself exerted cardioprotection versus the control group (27.2±1.3% *vs* 47.7±1.8%, p<0.05) (Figure 2C). Selective inhibition of iNOS did not abrogate the infarct limiting effects of NTG (20.3±2.8%, p=NS vs NTG group), whereas 1400W reduced the %I/R versus the control group (15.0±0.6% *vs* 47.7±1.8%, p<0.05,) as expected[350]. eNOS was phosphorylated at ser 1176 after NTG treatment, an effect that was inhibited after L-NAME administration (Figure 2D).

Wortmannin, and L-NAME administration did not significantly change infarct size compared to the control group (48.0±2.4%, and 37.7±1.8%, respectively, p=NS) (Figures 1C, 2C).

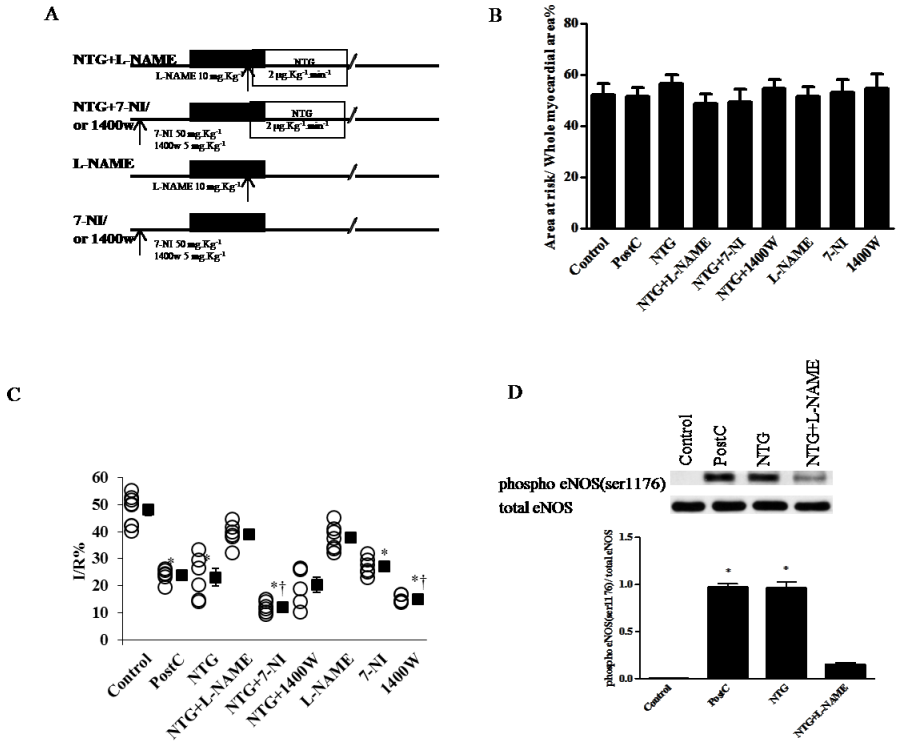


Figure 2. **NTG phosphorylates eNOS.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative blots for peNOS/teNOS are shown along with the densitometric analysis from the total number of animals per group (n=5) (*p<0,05 versus Control group, †p<0,05 versus PostC and NTG groups)

Furthermore, the addition of the PKG inhibitor DT2 did not abrogated the infarct size limiting effect of NTG (19.5±2.5 %, p=NS *vs* NTG) (Figure 3B, 3C).

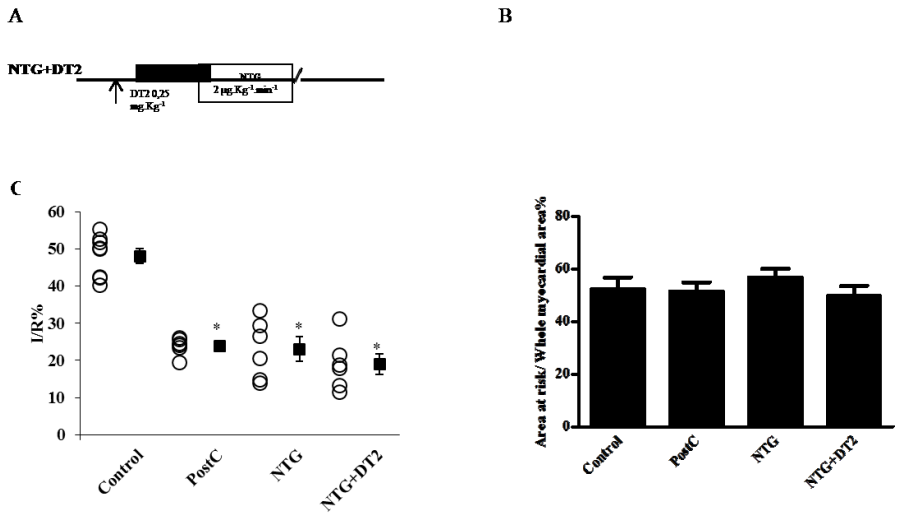


Figure 3. **NTG does not activate PKG.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R). (*p<0,05 versus Control group)

2.4.3. NTG reduces myocardial infarct size in the murine myocardium. Evidence for a species specific eNOS phosphorylation mechanism.

To extend our findings in a different animal model, as eNOS cardioprotective contribution has been shown to differ among mammals[345] we administrated NTG in the murine model. In mice under ischemia/ reperfusion no significant differences were detected in the areas at risk among groups (Figure 4B). It is well known that NTG induces nitrate tolerance both in the vascularate and in cardiomyocytes which abrogates its beneficial effects in the cardiovascular system[240, 241, 343]; in order to define the effects of acute administration of NTG in our model we included as positive control a well described model of NTG administration that induces nitrate tolerance (NTG tolerance group)[347] in the murine heart. As it was expected NTG tolerance group developed a myocardial infarction similar to the one of the Control group (46.09±3.3% and 53.3±2.1%, respectively). Acute administration of NTG in the murine myocardium reduced myocardial infarct size compared to the control and NTG tolerance groups (23.2±2.7% vs 53.3±2.1% and 46.09±3.3% respectively, $p<0.05$), (Figure 4C, 4D). However, eNOS phosphorylation at ser1176 was not increased in the murine heart, in contrast to the increased phospshorylation that was observed in the rabbit heart (Figure 4E).

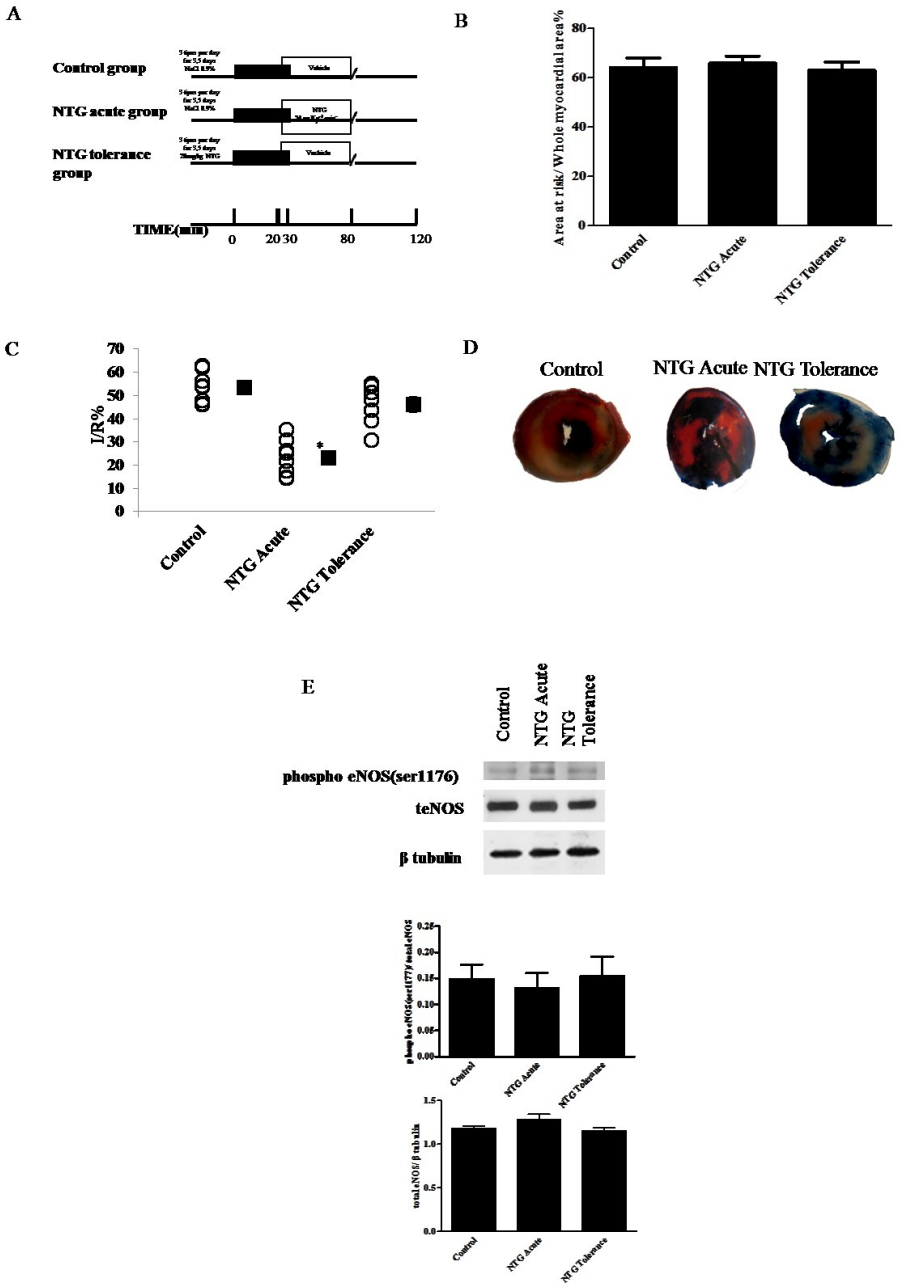


Figure 4. **Species specific eNOS phosphorylation by NTG.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative photos of the murine hearts, E. Representative blots for peNOS/teNOS are shown along with the densitometric analysis from the total number of animals per group (n=5) (*p<0,05 versus Control group)

2.4.4. Reduced nitro-oxidative stress seems the dominant mechanism of cardioprotection afforded by NTG

Direct reactive oxygen species assessment by DHE staining revealed reduced reactive oxygen species formation in the ischemic tissue of NTG treated animals in both models (Figures 5A, 5B, 5C) and the observed effect on ROS levels was driven in a NOS dependent manner, as co-administration of NTG and L-NAME resulted in increased formation of ROS in rabbit myocardium (Figure 5A). Acute administration of NTG resulted in decreased formation of ROS compared to the Control and to NTG tolerance groups in murine myocardium during reperfusion (Figure 5B). The formation of ROS was more evident in the ischemic part of the myocardium (Fig. 5C).

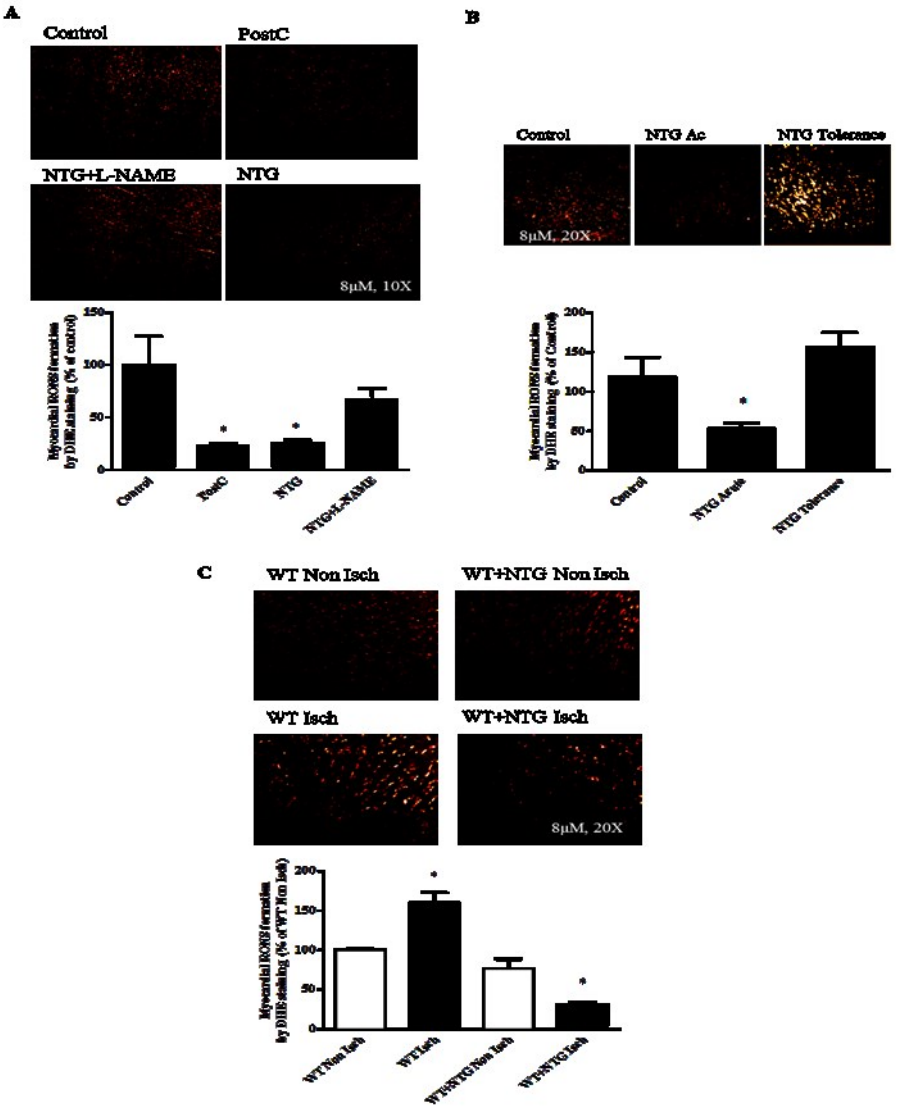


Figure 5. **NTG reduces nitro-oxidative stress in a NOS dependent manner.** A. Representative DHE staining in the rabbit ischemic heart along with the densitometric analysis, B;C. Representative DHE staining in the murine ischemic heart along with the densitometric analysis. (*p<0,05 versus Control group)

2.4.5. Genetic evidence for eNOS dependent NTG protection

To evaluate the role of eNOS activation in the murine heart and to investigate the role of eNOS activation by NTG in the different animal models we used wild type and eNOS(-/-) mice under vehicle or NTG treatment. No significant differences were detected in the areas at risk among the studied groups (Figure 6B). The infarct size was in line with previous findings[345] in Control wild type mice and it was similar to that of eNOS(-/-) animals (53.3 ± 3.2% vs 51.4± 3.5%, p=NS). However the infarct limiting effects of NTG were preserved only in the wild type compared to the eNOS(-/-) mice (21.7 ± 3.3% *vs* 46.4 ± 2.9% p<0.05) (Figure 6C, 6D).

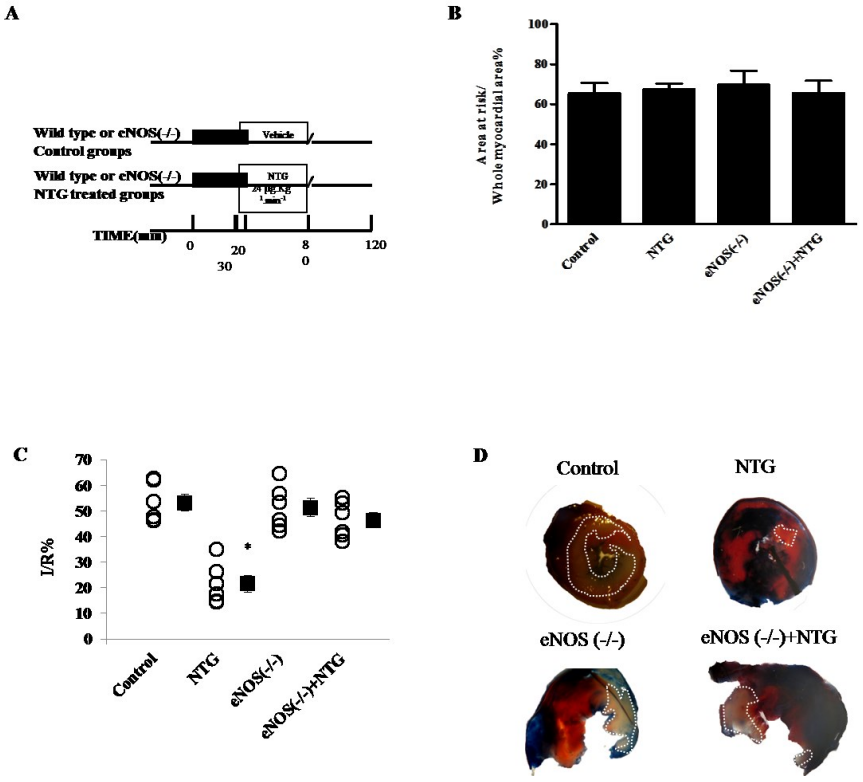


Figure 6. **Ablation of eNOS abrogates the NTG infarct limiting effects.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative photos of the murine hearts, (*p<0,05 versus wild type control, eNOS(-/-) and eNOS(-/-) +NTG groups).

2.4.6. Genetic evidence for CypD dependent NTG protection

To evaluate the role of mPTP pores opening by NTG we used wild type and CypD(-/-) mice under vehicle or NTG treatment. No significant differences were detected in the areas at risk among the studied groups (Figure 8B). The infarct size in CypD(-/-) mice was lower than that of the wild type animals (19.2 ± 1.9% vs 51.4± 3.5%, p<0.05), a finding that it was in line with previous findings (Bell et al., BRC 2013). Administration of NTG in CypD(-/-) mice had no additional effect on infarction limitation (18.6 ± 1.8%) (Figure 8C, 8D).

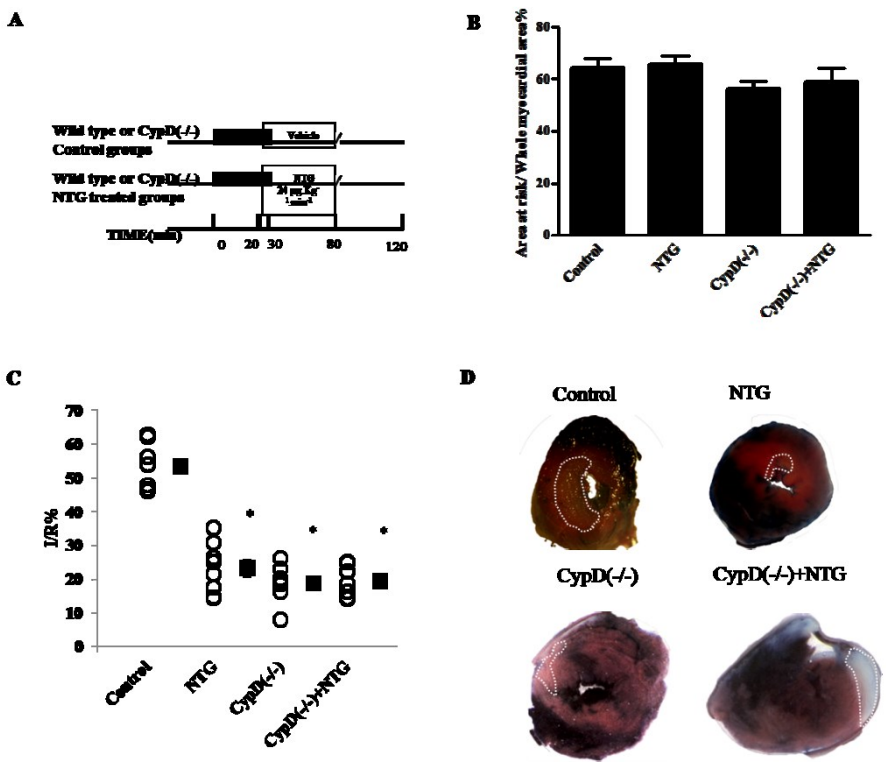


Figure 8. ***NTG confers cardioprotection in a CypD dependent manner.*** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative photos of the murine hearts, (*p<0,05 versus wild type control).

2.4.7. NTG preserves its beneficial effects in a model of endothelial dysfunction

To assess the effect of NTG in a model of endothelial dysfunction we used ApoE (-/-) mice under vehicle or NTG treatment. The infarct size in ApoE(-/-) mice was similar to that of the wild type animals (49.7 ± 4.3% vs 51.4± 3.5%, p=NS). Administration of NTG in ApoE(-/-) mice preserved its beneficial effects compared to the wild type animals (20.0 ± 3.6%) (Figure 9B, 9D). No significant differences were detected in the areas at risk among the studied groups (Figure 9C).

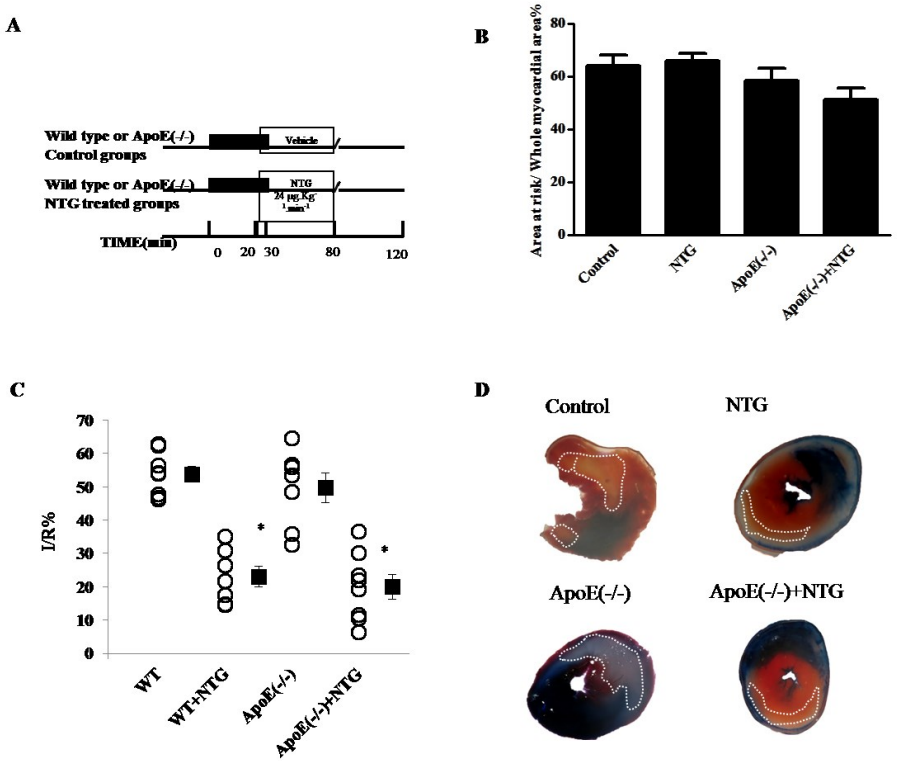


Figure 9. **NTG preserves its infarct limiting effects in ApoE KO animals.** A.Schematic presentation of the study protocol B. Area at risk to whole myocardial area ratio (AAR/A %), C. Infarct size to area at risk ratio (%I/R), D. Representative photos of the murine hearts, (*p<0,05 versus).

2.5. Discussion

The present study indicates that an acute, non- hypotensive NTG administration during evolving myocardial infarction is cardioprotective against ischemia and reperfusion injury in in vivo models. We present for the first time, i. a novel mechanism of NTG, which encompasses the dominant role of eNOS, reduced oxidative stress and inhibition of mPTP opening, explaining the infarct size reduction, ii. that NTG is capable of minimizing cardiac death even under endothelial impairment conditions.

Low doses of nitroglycerin (less than 30 micrograms/min) intravenously (IV) given, act as a dominant venodilator, while higher doses balance between venous and arterial dilation[342]. Studies in canine models have demonstrated that in the early stages of infarction, low-dose NTG infusion, carefully titrated for only minimal mean blood pressure reduction, markedly reduces left ventricular (LV) preload, improves regional perfusion, and reduces infarct size and adverse remodeling. However, higher doses of IV NTG decrease mean blood pressure, causing hypoperfusion and increasing the final infarct size[351]. These results were replicated in humans as lower NTG dosage protocol may have the advantages of prolonging the beneficial effects by lowering left ventricular filling pressure and systemic vascular resistance in patients with acute myocardial infarction[342], allowing more precise control of dosage, and thus avoiding sudden falls in arterial pressure. Clinical studies have confirmed that low-dose NTG IV infusion for the first 48 hours after acute myocardial infarction is safe, not only for improving LV performance but also for limiting ischemic injury, infarct size, adverse remodeling, and complications related to infarction, including in-hospital and one year follow-up deaths[351-354]. As shown in patients with anterior AMI[355], IV NTG is associated with reversal of restrictive left ventricular diastolic filling pattern in the early stages of AMI. However, although studies in humans support an infarct size limiting effect of NTG if it is given at the time of evolving infarction, the AHA guidelines for AMI patients, challenge its treatment benefits which are limited and no conclusive for routine IV, oral, or topical use. Thus, studies that will emerge a protective role of a non hypotensive dose of NTG in evolving AMI, and will elucidate the underlying molecular mechanism of action in the infarcted myocardium would probably lead to the establishment of NTG as a first line drug in the limitation of myocardial infarct size.

Mechanistically, endogenous NO plays a predominant role in conditioning strategies, as once bioavailable it minimizes myocardial infarct size[90, 180, 356-358]. Therefore, a number of studies tried to mimic endogenous NO beneficial effects by administrating NO releasing molecules prior to the ischemic insult[41, 83, 359, 360]. However, a limited number of studies have reported a protective role when NO releasing drugs are administered in the clinical setting during the ischemic insult.

NTG's mode of action has received considerable attention for several decades. Despite the extensive and well described vasodilatory effects as well as adverse effects upon chronic administration (e.g. oxidative stress and nitrate tolerance)[361], only small progress has been achieved regarding its cardioprotective effects and its mode of action. Controversial findings regarding the reduction of myocardial necrosis after the administration of a non-hypotensive NTG dose prior to reperfusion have been reported so far [245-247]. NTG minimizes myocardial infarction *in vivo* in dogs[247], whilst it has not been proved to be cardioprotective when it is administered during ischemia in anesthetized pigs[245] and rabbits[246]. In our study, NTG given at the end of the ischemia and during reperfusion, at a dose which does not change haemodynamic parameters, thus, reflecting the routine clinical practice[362], reduces infarct size

in anesthetized rabbits and mice. The divergent results between the others and the present study may be due to i) the observed haemodynamic instability in the study of Liu et al[245]., which suggests a progressive decline of LV function upon NTG administration in their model and ii) dosage protocol in the latter study of Salloum et al[246]., in which a constant rate of 50 ml/hr for 65 min was used. In our study the dosage protocol was 2 μg kg^{-1} min^{-1} at a constant rate of 1 ml/h for 65 min starting at 10 min prior to the onset of reperfusion. Guidelines deduce that optimal administration volume, shall be <10% of the circulating blood volume of the animal. Considering that the plasma volume of rabbit is approximately 55.3 $\pm$ 5.3 ml· kg^{-1}, [363] this leads to the conclusion that volumes greater than 8 ml/hr are not well tolerated and cause serious volume increase[364].

In terms of the molecular mechanism of NTG action, no studies have been performed so far for its cardioprotective effects, whereas the most of the studies have focused on the molecular mechanism which mediates the vasodilatpry effects of NTG. Therefore, there are studies suggesting an eNOS dependent signaling of NTG's vasodilatory effects. It has been reported that the vasodilatory effects of NTG are driven through accumulation of 3,4,5-$InsP_3$, probably because of inhibition of PTEN, and further activation of PI3K/Akt and eNOS[365]. In agreement to the above, we observed that inhibition of PI3K by Wortmannin reversed NTG's cardioprotective effects; furthermore, inhibition of NOS by L-NAME completely abrogated the beneficial effects of NTG. Thus, in order to further investigate the NOS isoform responsible for the cardioprotection observed, we selectively inhibited nNOS and iNOS. We should mention that the role of nNOS[67, 366] and iNOS[350, 367] in cardioprotection has led to divergent results in the literature. Our results showed that nNOS inhibition resulted in infarct size limitation as previously described in brain ischemia reperfusion injury[368], while co-administration of NTG led to a further reduction in infarct size, indicating that the cardioprotective effects afforded by NTG are independent of nNOS activation. Similar results were observed for iNOS inhibition, as NTG administration along with inhibition of iNOS did not reverse NTG's beneficial effects. Taken together, our results indicate that eNOS isoform plays a key role for NTG–mediated cardioprotection.

As we mentioned before, eNOS is critically involved in the amplification of the vasodilator effects elicited by low dose NTG[369]. However, this report has raised contradictory responses by other groups[370]. Furthermore, other reports showed an endothelium dependence of NTG effects in animals and in patients[371-373]. L-arginine, a nitric oxide synthase substrate, is capable of amplifying and sustaining NTG-induced NO production[374]. However, although there are studies which support the role of eNOS on NTG's induced vasorelaxation, there are no studies until now investigating its role in cardioprotection. To shed some light on this issue and to clarify if eNOS was mandatory to NTG induced cardioprotection, as indicated by our *in vivo* rabbit cohort results, we evaluated NTG infarct limiting effects in eNOS knockout mice. We observed that in contrast to the wild type animals, in which NTG reduced infarct size, eNOS knock out animals were not protected. However, it has been reported that NTG is effective in eNOS knock out animals concerning its vasodilatory effects; this was mainly explained by the fact that eNOS knock out animals over express nNOS[375], which is further activated by NTG in mice aorta[369]. Although this might be the case for the vasodilatory effects of NTG, we found that ablation of eNOS completely abrogated NTG's effects on infarct size limitation. This discrepancy possibly is due to the fact that NTG's infarct size-limiting effects are independent of nNOS, as we observed in the rabbit cohort.

Taken for granted that eNOS is mandatory for NTG beneficial effects, we evaluated the possibility that NTG administration could increase eNOS activity by inducing its phosphorylation on the activatory Ser1177. We observed controversial results, as eNOS was found phosphorylated in the rabbit ischemic heart, however, in the murine ischemic heart no phosphorylation of Ser1177 was observed. Although in the literature concerning the vasodilatory effects of NTG, it has been reported that eNOS phosphorylation increases[365]. These observations are in line with the fact that NTG exposure in cultured endothelial cells led to the accumulation of citrulline, indicative of NOS activation[376] .However, the discrepancy observed cannot be explained by the present data. Further studies are needed to evaluate other phosphorylation sites of eNOS. Perhaps a role of PYK2 kinase which is activate under oxidative stress and leads to a dual phosphorylation of eNOS (as described in experimental study I) is the key to the explanation.

Our next step was to investigate the downstream pathway following eNOS. The eNOS/ PKG pathway has been linked to PostC[91, 345] and recently it has been confirmed that activation of the cGMP/PKG pathway depends on NOS but is independent of PI3K/Akt signaling[91]. To investigate if PKG was activated following eNOS activation by NTG we co-administrated a PKG-I inhibitor, the compound DT-2. We observed that blockade of PKG-I preserved NTG's cardioprotective effects. This finding is in line with the observations of Cohen et al., 2010 who suggested an NO-dependent but PKG-independent protection following administration of the NO donor SNAP in isolated rabbit heart[164]. Additionally, it has been proposed that during reperfusion NO acts directly in limiting infarct size independently of PKG activation by increasing protein nitrosylation by 25%[41, 358]. Our data reinforce this notion of a PKG independent protection, and strengthen the hypothesis of the second eNOS target during reperfusion, in an *in vivo* model.

The produced NO at the beginning of reperfusion has been proposed to react with ROS and form the highly toxic peroxynitrite, which is responsible for a further increase of ROS through eNOS uncoupling leading to increased nitro-oxidative stress and cell death[377, 378]. Thus, we further investigated the effects of NTG treatment on myocardial nitro-oxidative stress. We demonstrated that ROS levels in the ischemic myocardium of both models were reduced upon NTG treatment; whilst were increased by NOS inhibition, indicating an eNOS dependent antioxidant role of NTG in the rabbit model. In addition, minimized ROS formation was observed also in the acute low dose NTG treated murine heart. This is in line with the observation that intravenous NTG reduces lipid peroxidation during ischemia-reperfusion injury in humans during AMI[379]. Reviewing the current state of the art, it has been shown in preclinical studies that the presence of nitrate tolerance aggravates ischemia/reperfusion injury and leads to loss of the cardioprotective effect of PostC possibly due to increased systemic formation of peroxynitrite[380]. In addition, in recent clinical studies[242, 243], it has been reported that the endothelial preconditioning effect of a single dose of NTG is lost upon a prolonged exposure to NTG as well as repeated short-term exposures (2h/d for 6d or 7d). This is in agreement with our findings in murine myocardium whereas increased nitro-oxidative stress was observed in NTG tolerance group. Taken together with our results, NTG is an effective cardioprotective agent when it is administered short term, as it does not impair endothelium but in turn reduces reactive oxygen species in both rabbits and mice.

There is little information regarding the molecular mechanisms underlying the effect of NO in PostC. In general, it is believed that PostC acts by inhibition of the mitochondrial

permeability transition pore (mPTP) opening. NO prevents the opening of mPTP and inhibits the cardiac mitochondrial voltage-dependent anion channel (VDAC)[42]. NO endorses its properties on target proteins by two signal pathways: cGMP-dependent pathway and redox-related protein modification, the latter being independent of cGMP. In the cGMP-dependent pathway, NO modulates the activity of PKG, whereas PKG alters the function of targeted proteins[43]. The redox-related protein modifications induced by NO pathway include S-nitros(yl)ation, nitration, and oxidation. These posttranslational protein modifications are dependent on the redox state[44]. It has been demonstrated that exogenously applied NO had diverse and concentration-dependent effects on mPTP opening. The high concentrations of NO opened mPTP and these effects were related to both disulfide bonds and $ONOO^-$ formation. In contrast, physiological concentrations of NO inhibited mPTP opening by modifying thiol residues possibly through S-nitros(yl)ation[42]. In our study we showed that the cardioprotective effects of NTG are independent of PKG activation. It has been referred that the modification of cysteine thiol residues caused by low-concentration of NO may reduce the access of $ONOO^-$ to protein SH residues and prevent further oxidative modification[381]. This might be also the reason of the reduced nitro-oxidative stress that was observed in the ischemic myocardium treated with NTG at the beginning of reperfusion. Thus, the production of low, physiological concentrations of NO produced by NTG administration, further causes an increase in SNO and a decrease in nitro-oxidative stress.

In addition, direct NO cardioprotective effects have been attributed to inhibition of mPTP opening. Cyclophilin D (Cyp D), which accelerates mPTP opening, undergoes S-nitrosylation on cysteine 203 leading to reduced mPTP opening in mice wild type fibroblast but not in Cyp D knockout fibroblast[382]. Therefore, we tested if NTG was able to minimize myocardial infarct size in CypD KO mice. NTG administration on this KO model did not enhance the cardioprotective effects, indicating that NTG acts in a CypD dependent manner.

Finally, to further confirm the role of NTG in conditions of endothelial dysfunction, we administrated NTG in a model of reduced endogenous NO production. ApoE KO animals have been shown to exhibit endothelial impairment as they are characterized by increased superoxide formation and reduced NO release by uncoupled eNOS[383, 384]. We observed that ApoE KO animals were still protected upon NTG administration. This in combination with the fact that NTG is acting as an antioxidant agent concludes that it could be a potent cardioprotective agent in the presence of endothelial dysfunction conditions.

In conclusion, herein we demonstrate by findings in two *in vivo* models of myocardial infarction that low dose NTG reduces myocardial infarct size when administrated during the ischemic insult under both normal and endothelial dysfunction conditions. The mechanism of action in the ischemic heart relies on PI3K/Akt activation, inhibition of mPTP opening and reduced oxidative stress. eNOS presence is mandatory for NTG's induced cardioprotective effects and it seems to mediate the antioxidant effects of NTG. The exact mechanism by which the synthase is correlated to cardioprotection afforded by NTG is not clear and further studies will clarify this issue.

2.6. Clinical implications

Two of the major objectives in the therapy of acute myocardial infarction are preservation of borderline ischemic myocardium and thus reduction of infarct size, and improvement of left ventricular function in patients with evidence of left ventricular failure. Positive inotropic agents and volume expansion may improve left ventricular function but both treatments are detrimental and increase myocardial oxygen demand increasing and not decreasing the size of infarction. Reduction of left ventricular preload decreases left ventricular volume, wall tension, and therefore, myocardial oxygen demands. A decrease in afterload reduces oxygen demands and improves left ventricular function. However, a significant reduction of mean arterial pressure would obviously result in a reduction of coronary perfusion pressure and thus may cause larger myocardial infarction.

NTG has dose dependent effects. In low doses it acts as a venodilator thus reducing cardiac preload which potentially alleviates nutrients and oxygen demands of the ischemic heart [239]; however, in higher doses venous and arterial dilating effects are balanced, affecting myocardial perfusion and thus potentially increase infarct size[342]. AHA guidelines upon NTG use on AMI patients are complex. They suggest that no conclusive evidence has been shown to support the routine use of IV, oral, or topical nitrate therapy in patients with AMI. With this in mind, these agents should be carefully considered, especially in the patient with low blood pressure. Patients with ischemic discomfort should receive up to 3 doses of sublingual or aerosol nitroglycerin at 3- to 5-minute intervals until pain is relieved or low blood pressure limits its use. In patients with recurrent ischemia, nitrates are indicated in the first 24 to 48 hours. Administer nitrates with extreme caution, if at all, to patients with inferior-wall MI and suspected right ventricular (RV) involvement because these patients require adequate RV preload. Nitroglycerin in the doses indicated by AHA guidelines is more possible to lead to a precipitous fall in arterial pressure and a reflex tachycardia may occur. This could result in an increase in infarct size. Herein by findings supported in two *in vivo* animal models of myocardial infarction, we suggest that by titration of the infusion rate of NTG, elevated left ventricular filling pressure might fell to within normal limits and work in favor of infarct size limitation. The implication of eNOS and decrease of ROS formation in the ischemic myocardium as an underlying mechanism of NTG's cardioprotection may indicate its usefulness in patients with impaired endothelial function.

Experimental Study III:

Hydrogen sulfide induces cardioprotection.
The role of the NO/cGMP/PKG/PLN pathway.

3.1. Abstract

Aims H_2S is known to confer cardioprotection, however, the pathways mediating its effects *in vivo* remain incompletely understood. The purpose of the present study is to evaluate the contribution of cGMP-regulated pathways in the infarct-limiting effect of H_2S *in vivo*.

Methods and results Anesthetized rabbits were subjected to myocardial ischemia (I)/reperfusion (R) and infarct size was determined in control (c) or H_2S exposed groups. NaHS (an agent that generates H_2S) increased cardiac cGMP and reduced the infarct size. The cGMP-dependent protein kinase (PKG)-I inhibitor DT2 abrogated the protective effect of NaHS, while the control peptide TAT or L-nitroarginine methyl ester (L-NAME) did not alter the effect of NaHS. Moreover, the K_{ATP} channel inhibitor glibenclamide partially reversed the effects of NaHS, while inhibition of mitochondrial K_{ATP} did not modify the NaHS response. NaHS enhanced phosphorylation of phospholamban (PLN), in a PKG-dependent manner. To further investigate the role of PLN in H_2S-mediated cardioprotection, wild type and PLN KO mice underwent ischemia-reperfusion. NaHS did not exert cardioprotection in PLN KO mice. Unlike what was observed in rabbits, genetic or pharmacological inhibition of eNOS abolished the infarct-limiting effect of NaHS in mice.

Conclusions Our findings demonstrate i) that administration of NaHS induces cardioprotection via a cGMP/PKG/PLN pathway and ii) contribution of NO to the H_2S response is species-specific.

3.2. Aim

Hydrogen sulfide is an endogenously produced gaseous signaling molecule, with important roles in the regulation of cardiovascular function[385]. Endogenously produced or exogenously supplied H_2S has been reported to be cytoprotective during reperfusion injury *in vitro* or in isolated hearts[249-251, 385-392]. However, in most of the studies where H_2S was exogenously administered to trigger conditioning, H_2S donor compounds were not given in a manner that would be consistent with administration of a pharmacological agent in patients, as the donors were given hours to days prior to induction of ischemia. In the limited number of *in vivo* studies, administration of H_2S producing agents during sustained ischemia has been shown to be beneficial in infarct size limitation and in post-infarction myocardial function[33, 36-38]. This cytoprotection was associated with up-regulation of antioxidant pathways[36], inhibition of myocardial inflammation[37], preservation of mitochondrial structure and function[37] after ischemia and inhibition of apoptosis[37, 38]. Whilst the cardioprotective effects of H_2S have been reported to be nitric oxide (NO)-dependent, this was claimed to occur in a cGMP-independent manner[33]. In the studies where H_2S was administered at a clinically relevant time (at the end of ischemia and during reperfusion)[33, 36-38], the intracellular signalling pathways determining H_2S-induced cardioprotection were not studied in detail. The positive preclinical findings with H_2S donors, have served as the impetus for a clinical trial designed to evaluate the cardioprotective potential of H_2S in humans (NCT01989208).

Given the increasing interest on the role of H_2S, herein we aimed:

1. To investigate the cardioprotective effects of the rapid releasing H_2S donor, NaHS on myocardial infarction
2. To assess the possible exogenous H_2S- endogenous NO interplay in the cardioprotective effects of NaHS in the ischemic myocardium
3. To elucidate the possible involvement of cGMP/PKG pathway on the cardioprotective effects of NaHS.
4. To evaluate possible downstream targets on PKG activation (i.e. PLN) that their role has not yet been evaluated in detail in ischemic postconditioning pharmacological or not.

3.3. Materials and Methods

3.3.1. Animals

All animal procedures were in compliance with the European Community guidelines for the use of experimental animals; experimental protocols were approved by the Ethical Committee of the Prefecture of Athens. Animals received standard laboratory diet. The initial animal cohort compromised of 92 New Zealand white male rabbits 2,7-3 kg, 44 C57BL/6 male mice 13-15 weeks old, 12 eNOS KO male mice 13-15 weeks old, 29 129SvJ male and female mice 12-15 weeks old and 27 PLN KO male and female mice 12-15 weeks old.

3.3.2. Surgical Procedures

3.3.2.1. Rabbit in vivo model of ischemia/reperfusion injury

Described in Experimental Study II, section 2.3.2.1

3.3.2.2. Murine in Vivo Model of Ischemia-Reperfusion Injury

Described in Experimental Study I, section 1.3.12.1.

3.3.3. Chemicals

DT2: BIOLOG Life Science Institute (Bremen, Germany); Glibenclamibe: Tocris Bioscience (Bristol, United Kingdom); Phosphodiesterase activity colorimetric assay kit and TAT: Abcam (Cambridge, United Kingdom); L-NAME: Cayman CHEMICALS (Lab Supplies-P.Galanis & Co., Athens, Greece); NaHS and 5-hydroxydecanoate: SIGMA-ALDRICH Co. (Life Science Chemilab S.A., Athens, Greece); Protein G Sepharose Fast flow beads: GE Healthcare Life Sciences (G. Kordopatis Ltd, Athens, Greece).

3.3.4. Experimental protocol

3.3.4.1. Experiments in rabbits

New Zealand white male rabbits weighing 2.7-3.2 kg were subjected to 30 minutes regional ischemia of the myocardium, followed by 3 hours of reperfusion and were randomized into 10 groups as follows (experimental protocol presented in detail in Figure 1A):

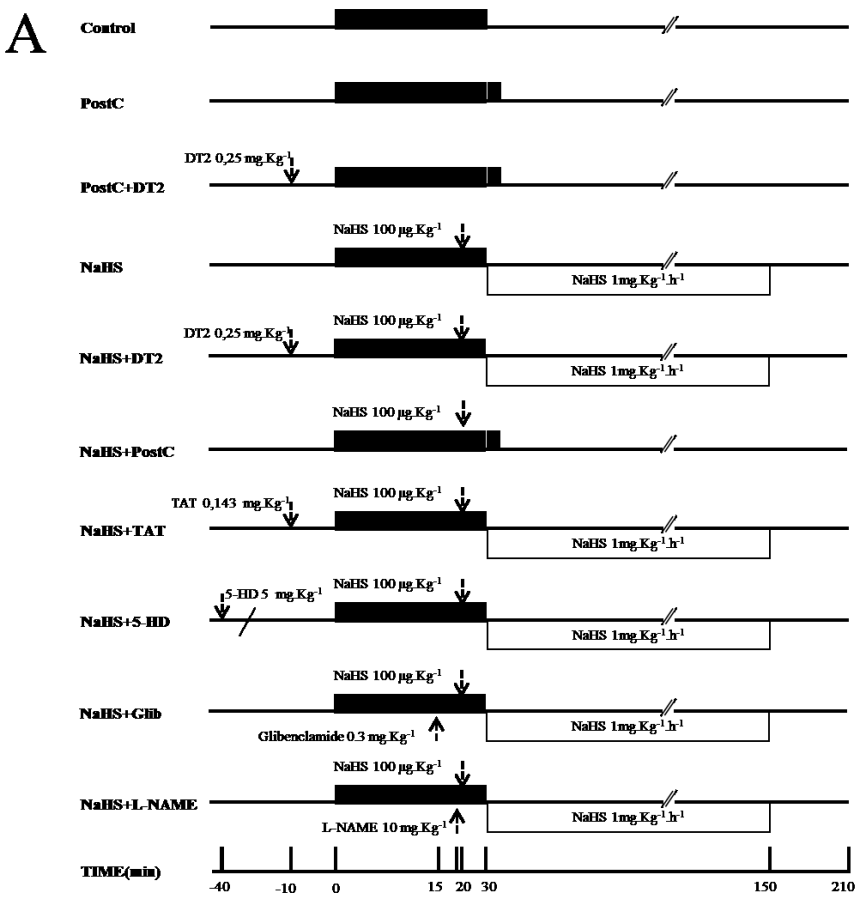


Figure 1A: **Experimental protocol of experimental series 1**

Control group (n=8): No additional intervention.

PostC group (n=7): Application of 8 cycles of 30 sec ischemia-reperfusion immediately after sustained ischemia.

PostC+DT2 group (n=6): Application of PostC and administration of the PKG inhibitor DT2 at a dose of 0.25 $mg \cdot kg^{-1}$ iv bolus 10 min before sustained ischemia, as previously described[393].

NaHS group (n=7): Animals were treated with the H_2S donor sodium hydrosulfide (NaHS) at a dose of 100 $\mu g \cdot Kg^{-1}$ iv bolus on the 20th min of ischemia followed by infusion of $1mg \cdot Kg^{-1} \cdot h^{-1}$ for the next 120 min, as previously described[36].

NaHS+DT2 group (n=6): Animals were treated with NaHS (as in the NaHS group) and the PKG inhibitor DT2 given at a dose of 0.25 $mg \cdot kg^{-1}$ iv bolus 10 min before sustained ischemia[393].

NaHS+TAT group (n=6): Animals were treated with NaHS (as in the NaHS group) and the control peptide of DT-2;TAT given at a dose of 0.143 $mg \cdot kg^{-1}$ iv bolus 10 min before sustained ischemia.

NaHS+PostC (n=6): Animals were treated with NaHS at a dose of 100 $\mu g \cdot Kg^{-1}$ iv bolus on the 20th min of ischemia followed by 8 cycles of 30 sec ischemia-reperfusion immediately after sustained ischemia.

NaHS+5-HD group (n=10): Animals were treated with NaHS (as in the NaHS group) and the mitoK$_{ATP}$ channels inhibitor 5-hydroxydecanoic acid (5-HD) iv bolus 40 minutes before occlusion at a dose of 5 mg·kg^{-1} as previously described[394].

NaHS+Glibenclamide group (n=9): Animals were treated with NaHS (as in the NaHS group) and the mitoK$_{ATP}$ and sarcK$_{ATP}$ channels inhibitor glibenclamide iv bolus at the 15th min of ischemia at a dose of 0.3 mg·kg^{-1} iv as previously described[356].

NaHS+L-NAME (n=6): Animals were treated with NaHS (as in the NaHS group) and the inhibitor of the synthase of NO (L-NAME) IV bolus at the 19th min of ischemia at a dose of 10 mg·kg^{-1} as previously described[248].

In a second series of experiments, rabbits (5 per group of Control, PostC, NaHS and NaHS+DT2 groups) were subjected to the same interventions up to the 10th minute of reperfusion, when tissue samples from the ischemic area of myocardium were collected, snap-frozen in liquid nitrogen and stored at -80^{o}C for western blot analysis of VASP, Erk1/2, GSK3β, eNOS and PLN.

3.3.4.2. Experiments in Mice

Forty four wild type C57BL/6 male mice 13-15 weeks old and twelve eNOS KO male mice 13-15 weeks old were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions (Figure 1B).

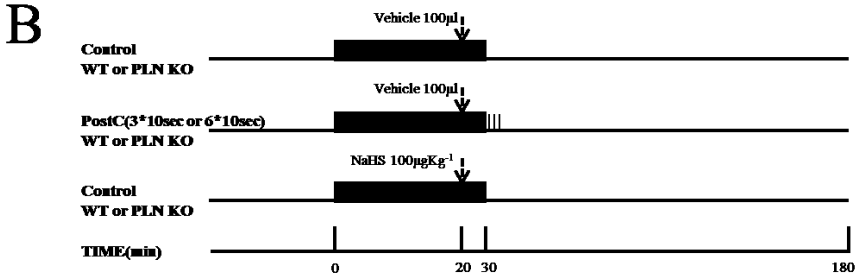


Figure 1B: **Experimental protocol of series 2**

Wild type group (n=6): Vehicle (100µl water for injection) administered iv at the 19th mine of ischemia.

Wild type group + NaHS (n=6): Administration of NaHS as an iv bolus dose of 100µg·kg^{-1} at the 20th min of ischemia as previously described[388].

Wild type group + DT2 + NaHS (n=6): Co-administration of NaHS (as described above) plus DT2 given at a dose of 0.37 mg·kg^{-1} iv bolus 10 min before sustained ischemia[393].

Wild type group + L-NAME (n=6): Administration of L-NAME as an iv bolus dose of 15mg·kg^{-1} at the 19th min of ischemia[248].

Wild type + NaHS + L-NAME group (n=6): Co-administration of NaHS plus L-NAME as described above.

eNOS KO group (n=6): Vehicle (100µl water for injection) administered iv at the 19th min of ischemia.

eNOS KO + NaHS group (n=6): Administration of NaHS as an iv bolus dose of 100μg·kg^{-1} at the 20th min of ischemia[388].

In another series of experiments, WT mice (4 per group of Control, NaHS and NaHS+DT2 groups) were subjected to the same interventions up to the 10th minute of reperfusion, when tissue samples from the ischemic area of myocardium were collected, snap-frozen in liquid nitrogen and stored at -80^{o}C for western blot analysis of pVASP.

In another series of experiments, twenty nine wild type 129SvJ and twenty seven PLN knockout male and female mice were subjected to 30 min regional ischemia of the myocardium followed by 2 hours of reperfusion with the following interventions (Figure 1C):

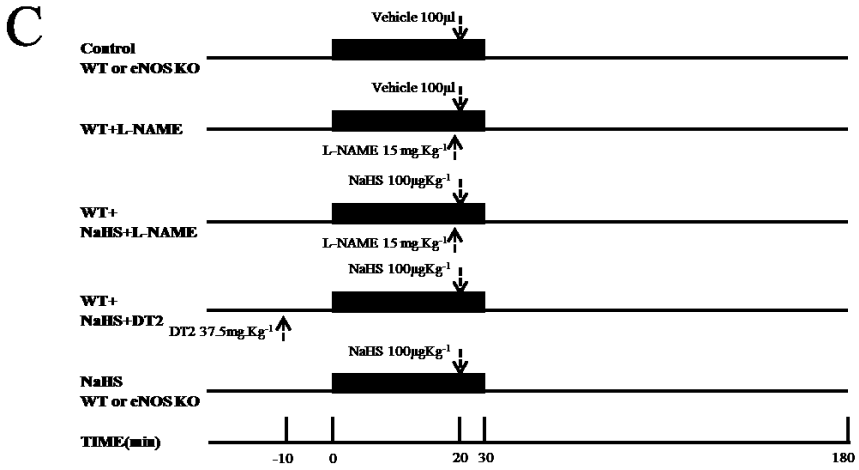


Figure 1C: **Experimental protocol of series 3**

Wild type group (n=8): Vehicle (100μl water for injection) administered iv at the 20th min of ischemia.

Wild type + PostC 3*10sec group (n=7): Application of 3 cycles of 10 seconds ischemia followed by 10 seconds of reperfusion immediately after prolonged ischemia[187].

Wild type+ PostC 6*10sec group (n=6): Application of 6 cycles of 10 seconds ischemia followed by 10 seconds of reperfusion immediately after prolonged ischemia[187].

Wild type + NaHS group (n=8): Administration of NaHS iv as a bolus dose of 100μg·kg^{-1} at the 20th min of ischemia[388].

PLN KO group (n=6): Vehicle (100μl water for injection) administered iv at the 20th min of ischemia.

PLN KO + PostC 3*10 sec group (n=9): Application of 3 cycles of 10 seconds ischemia followed by 10 seconds of reperfusion immediately after prolonged ischemia

PLN KO + PostC 6*10sec group (n=6): Application of 6 cycles of 10 seconds ischemia followed by 10 seconds of reperfusion immediately after prolonged ischemia[187].

PLN KO + NaHS group (n=6): Administration of NaHS iv as a bolus dose of 100μg·kg^{-1} at the 20th min of ischemia[388].

3.3.5. Western Blot Analysis

Described in Experimental Study I, section 1.3.4.

The following primary antibodies were used in the present study: phospho eNOS (ser1176), eNOS, phospho Akt(ser473), Akt, phospho VASP (Ser239), VASP, phospho GSK3β(ser9), GSK3β, phospho PLN(ser16/tyr17), PLN, phospho p44/p42, p44/p42,beta tubulin, beta actin (Cell Signaling Technology, Beverly, MA, USA).

3.3.6. cGMP determination

Described in Experimental Study I, section 1.3.11.

3.3.7. Phosphodiesterase activity

Rabbit left ventricular (LV) tissue homogenized in buffer containing 10 mM Tris-HCl (pH 7.4) in the presence of protease inhibitors. The homogenates were centrifuged at 15,000 x g (10 min) and supernatants were desalted by gel filtration. PDE activity was measured following manufacturer instruction. Briefly, 5 μg of protein was added to each well containing 3',5'-cGMP as substrate, with or without a H_2S donor, and incubated at 37°C for 30 min. The reaction was terminated by adding green assay reagent and color was allowed to develop for 30 min. Absorbance at 630 nm was read in a GENios micro-plate reader (Tecan). PDE activity was calculated using a 5'-GMP standard curve.

3.3.8. Statistical analysis

All results are presented as mean ± standard error mean. Comparisons of numeric variables among the groups were analyzed using one-way analysis of variance model (ANOVA) with Bonferroni correction and with Tukey post-hoc analysis. A two- or three-way ANOVA with Bonferroni correction to adjust for multiple pair-wise comparisons was employed for Figures 1, 4 and 5A. Analyses were performed using Stata 13.1 statistical software package (StataCorp, TX, USA). A calculated p value of less than 0.05 was considered to be statistically significant.

3.4. Results

3.4.1. Hemodynamic parameters

Characteristics and hemodynamic variables for all study rabbit groups are presented in Table 1. No significant differences were observed between the groups.

Table 1. Characteristics and hemodynamic variables for the different study group

	HW	**Baseline**		**20min Ischemia**		**180min Reperfusion**	
Study group		**HR**	**MAP**	**HR**	**MAP**	**HR**	**MAP**
Control	7.3±0.3	275±10	85±5	270±12	81±6	266±11	77±5
PostC	7.2±0.2	280±14	78±6	273±13	76±5	269±10	72±6
PostC+DT2	7.4±0.4	278±12	82±4	271±12	79±4	268±12	76±4
NaHS	7.8±0.3	282±11	84±3	274±09	80±3	270±11	77±7
NaHS+DT2	7.3±0.5	279±10	88±6	269±11	82±7	263±09	79±5
NaHS+TAT	7.4±0.4	274±09	81±7	267±10	77±6	265±13	74±3
NaHS+5HD	7.6±0.3	283±14	84±4	272±13	79±4	269±12	75±4
NaHS+Glibenclamide	7.7±0.2	271±15	79±6	267±11	75±5	264±14	73±5
NaHS+L-NAME	7.5±0.4	279±12	82±5	272±10	78±4	268±11	74±3

HW: Heart weight in g, HR: Mean heart rate in beats/min, MAP: Mean arterial blood pressure in mmHg

3.4.2. Infarct size determination in rabbits following pharmacological manipulations

To evaluate the effects of NaHS on cardioprotection, we used a rabbit *in vivo* model of ischemia-reperfusion injury. The rabbit exhibits a larger heart and slower heart rate than the mouse. In addition, the rabbit heart expresses the slow ß-myosin heavy chain isoform (MyHC), similar to the human heart, and its calcium cycling characteristics are similar to those of the human heart. Specifically, the rabbit and human heart share a major similarity in their reliance on SERCA and the sodium-calcium exchanger (NCX) for the extrusion of calcium during diastole[395].

In the rabbit cohort, no significant differences were detected in the areas at risk among the studied groups (Figure 2A). Application of PostC (as a positive control) limited infarct size compared to control (24.6±1.2% vs 47.5±1.2%, $p<0.05$), as expected (Figure 2B). NaHS

reduced the infarct size compared to control group (11.9±3.4% vs 47.5±1.2%, p<0.05) and did so to a greater extent than PostC (p<0.05).

DT2 is a membrane-permeable peptide which has the ability to inhibit cGMP-dependent PKG-Iα activation[186]. DT2 consists of peptide W45 (pseudosubstrate which cannot be phosphorylated by PKG-I) and a TAT peptide that is derived from the transactivator of transcription of HIV. TAT is a cell penetrating peptide allowing W45, or any other peptide sequence fused to it, to gain intracellular access. Although DT2 is a selective PKG-Iα inhibitor, the TAT peptide has been reported to exert biological effects of its own in vivo[87]. The addition of the PKG-Iα inhibitor DT2 abrogated the infarct size limiting effect of NaHS (39.8±3.5%, p=NS vs control), while TAT did not modify the effect of NaHS (23.1± 2.7%, p=NS vs NaHS group), suggesting that the effects of NaHS were PKG-Iα mediated. In addition, administration of DT2 along with PostC application reversed the beneficial effects of Po stC (42.6±1.8%, versus 24.6±1.2% in PostC, p<0.05).

Administration of the mitoK_{ATP} inhibitor (5-HD) did not affect the NaHS response (14.8±1.7%, p=NS). However, the mito/sarc K_{ATP} channel inhibitor glibenclamide, partially reversed the cardioprotective effects of NaHS (30.7±1.1, p<0.05 vs control and NaHS groups). Administration of pharmacological agents alone, (glibenclamide, 5-HD, L-NAME) in rabbits under ischemia/reperfusion, have been shown not to reduce infarct size (data not shown)[356]. In addition, application of PostC along with NaHS administration did not exhibit additional benefits to the ischemic myocardium compared to NaHS alone (9.5±1.2% vs 11.9±3.4%, p<0.05).

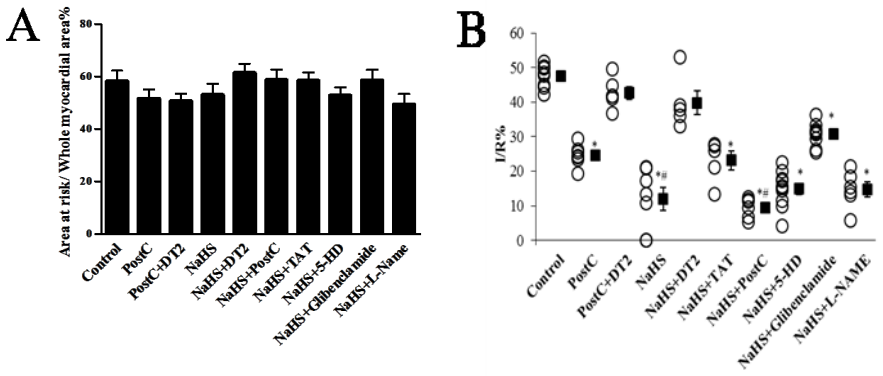


Figure 2: **Mechanism of NaHS-induced reduction in infarct size in rabbits**. Infarct area to area at risk ratio (%I/R) in rabbits. (*p<0.05 versus Control group, #p<0.05 versus PostC group). PostC (PostConditioning), DT2 (PKG-I inhibitor), TAT (control peptide of DT2), 5-HD (mitoK_{ATP} channels inhibitor), Glibenclamide (mito/sarc K_{ATP} channels inhibitor), L-NAME (NOS inhibitor).

3.4.3. Mechanisms of NaHS-induced pharmacological conditioning

To investigate whether NaHS administration results in increased cGMP levels in the heart, the levels of this cyclic nucleotide in the ischemic part of rabbit myocardium were measured

(Figure 3). In agreement with the results on infarct size suggesting involvement of PKG in the cardioprotective mechanism of H_2S, cGMP levels in the myocardium were found to be higher in NaHS-treated animals compared to the control group (Figure 3). The increase in cGMP levels coincided with inhibition of phosphodieasterase activity in rabbit heart homogenates; addition of 100μM of H_2S donor reduced cGMP-inhibiting PDE activity to 65±2.2% of control ($p<0.05$).

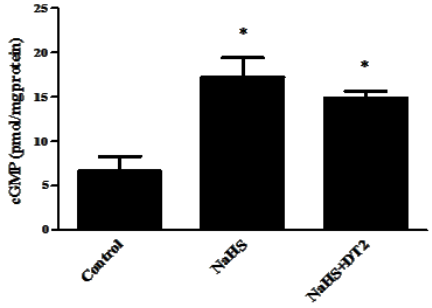


Figure 3: **cGMP levels in heart homogenates**. Following treatment, the ischemic part of the heart was snap-frozen, pulverized and cyclic nucleotides were extracted as detailed in the methods. Results are expressed as pmol/mg protein. (*$p<0,05$ versus Control)

Activation of PKG after NaHS treatment and PostC was confirmed by determining the phosphorylation status of its surrogate marker VASP. Indeed, VASP phosphorylation on Ser239 was greater in NaHS-treated animals compared to control and NaHS+DT2 groups (Figure 4A). Phosphorylation of Erk1/2 was also found significantly higher in NaHS and PostC groups versus control and NaHS+DT2 groups (Figure 4B), whereas no phosphorylation of GSK3β was observed in NaHS-treated animals; when PostC was used as a positive control an increase in GSK3β phosphorylation was noted, in line with reports in the literature[169] (Figure 4C). Since phospholamban (PLN) is a PKG substrate[170], we evaluated the ability of NaHS to alter its phosphorylation status. Levels of phospho-PLN were increased in the NaHS-treated group compared to control and NaHS+DT2 groups (Figure 4D). Similarly, PostC enhanced PLN phosphorylation.

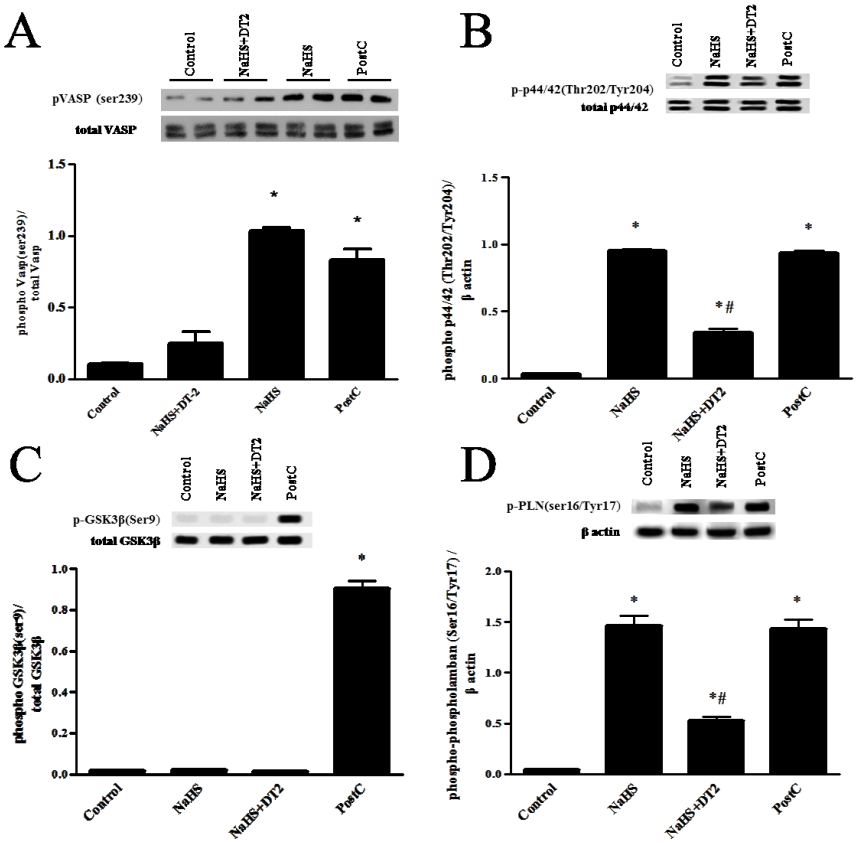


Figure 4: **PLN phosphorylation is PKG-dependent.** Representative blots for each signalling protein are shown along with the densitometric analysis from the total number of animals per group (n=5). (A) ratio of pVASP/VASP; (B) ratio of p-p44/42 / p44/42 (C) ratio of pGSK3β/GSK3β (D) ratio of pPLN/beta actin (*p<0.05 versus Control;#p<0.05 vs NaHS and PostC). PostC (PostConditioning), DT2 (PKG-I inhibitor).

3.4.4. Genetic evidence for PLN involvement in NaHS-induced protection

To evaluate the functional relevance of our biochemical findings on PLN, we used mice with targeted disruption of the PLN locus (Figure 5A). The infarct size in control wild-type mice (129SvJ background) was comparable to that of PLN KO animals (42.2±2.6% vs 41.3±3.1%, p=NS). PostC application and NaHS administration were beneficial in wild type animals compared to the control wild type group (14.9±2.2%, 15.5±1.1% vs 42.2±2.6% respectively, p<0.05). However, the cardioprotective effect of both NaHS and PostC was abolished in PLN KO animals (42.2±1.5%, 45.2±2.1% respectively, p<0.05 vs NaHS and PostC in wild-type mice). To test whether PLN KO mice require a more robust PostC algorithm we increased the PostC cycles. Applying 6 cycles of PostC did not lead to greater cardioprotection in wild-type mice, while in PLN KO animals we observed a trend towards a smaller infarct size that did not reach statistical significance. No significant differences were detected in the areas at risk among the studied groups (Figure 5B).

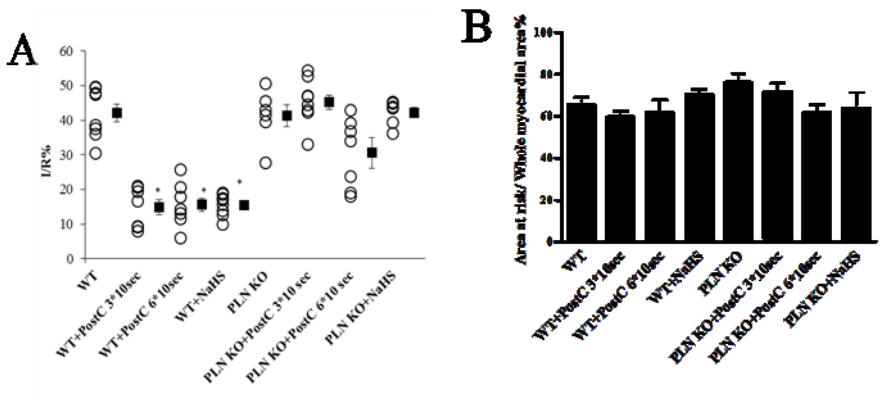
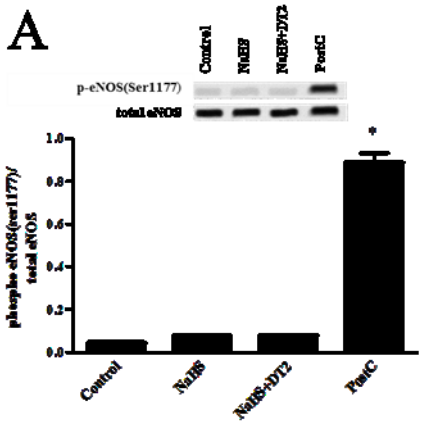


Figure 5: **NaHS-induced cardioprotection is PLN-dependent.** Infarct area to area at risk ratio (%I/R) in mice different study groups. (*p<0.05 versus Wild type, PLN KO, PLN KO+NaHS and PLN KO+PostC groups). PostC (PostConditioning).

3.4.5. Evidence of species-specific eNOS activation following NaHS-induced protection

In our initial experiments in rabbits, administration of the NOS inhibitor (L-NAME) did not alter the infarct limiting effects of NaHS (14.7±2.2% vs 11.9±3.4% respectively, p=NS vs NaHS) (Figure 2B). In addition, no phosphorylation of eNOS was observed in NaHS-treated animals, in contrast to what has been reported in the literature[33, 91, 228, 396, 397]. It should be noted that eNOS phosphorylation was evident in the positive control PostC group (Figure 6A). To reproduce the published results on the role of NO in H_2S cardioprotection in mice, we co-administered NaHS and L-NAME in mice. Such treatment resulted in complete abrogation of the NaHS-infarct size limiting effects (52.1±3.5% versus 20.1±4.3% respectively, p<0.05), with animals that received NaHS+L-NAME presenting infarct size similar to wild type and wild type + L-NAME groups (52.7±4.7% and 50.1±3.7% respectively, p=NS). The pharmacological findings were confirmed in the eNOS KO mice, where administration of NaHS had no protective results versus vehicle-treated eNOS KO animals (49.9±3.6% vs 51.4±3.5% respectively, p=NS) (Figure 6B). No significant differences were detected in the areas at risk among the studied groups (6C).



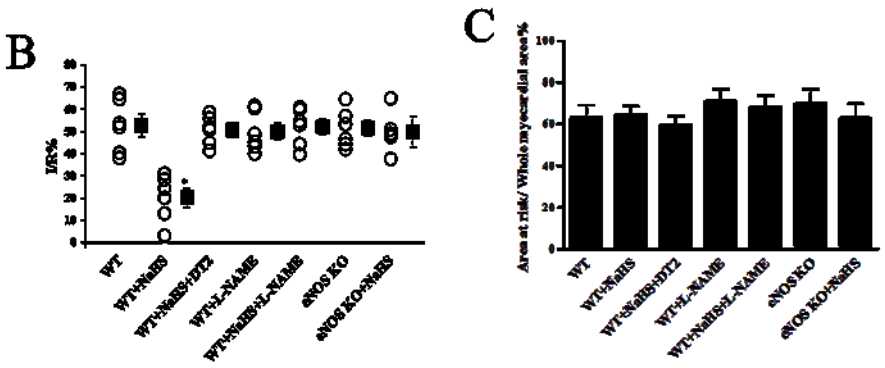


Figure 6. Inhibition of eNOS abolishes NaHS-induced cardioprotection in mice. A. Representative western blot and densidometric analysis ratio of peNOS/eNOS. (*p<0.05 versus Control;#p<0.05 vs NaHS and PostC). PostC (PostConditioning), DT2 (PKG-I inhibitor). B. Infarct area to area at risk ratio (%I/R) in mice. C. Area at risk to whole myocardial ares ratio (%). (*p<0.05 versus Wild type, L-NAME, eNOS KO+NaHS and eNOS KO groups). PostC (PostConditioning), L-NAME (NOS inhibitor).

3.4.6. PKG dependent cardioprotection of NaHS in murine myocardium

Since species differences were observed between rabbits and mice we sought to confirm the importance of PKG-Iα in cardioprotection in mice. Administration of a single iv bolus dose of NaHS in mice (C57Bl6 background) resulted in a significant reduction on infarct size compared to control group (20.1±4.3% versus 52.7±4.9% respectively, p<0.05). Inhibition of PKG-Iα before NaHS administration abrogated the infarct limiting effects of NaHS (50.4±2.8% versus 20.1±4.3% respectively, p<0.05) (Figure 6B). No significant differences were detected in the areas at risk among the studied groups (Figure 6C). Additionally, the administration of NaHS resulted in increased phosphorylation of VASP (ser239) compared to wild type control in the ischemic area of the myocardium at the 10th minute of reperfusion, whereas no phosphorylation was observed in the group treated with DT2 (Figure 7).

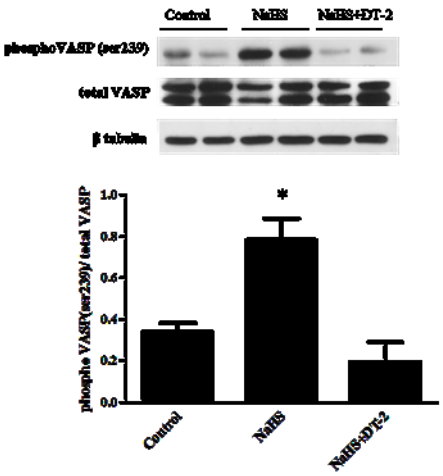


Figure 7: **PKG is activated in mice upon NaHS treatment.** Representative western blot and densidometric analysis of phospho VASP to total VASP ratio. *p<0.05 vs Control

3.5. Discussion

The current study confirms the cardioprotective effect of H_2S *in vivo* and highlights the importance of a novel cGMP/PKG/PLN signaling cascade in the pharmacological PostConditioning afforded by H_2S. Several studies have suggested a beneficial role of cGMP/PKG-regulated pathways in PostC[87, 91, 166, 228]. PKG activation exerts anti-apoptotic effects through PKCε activation and inhibition of mPTP channel opening[87]. In addition, increased cGMP levels and PKG activation lead to attenuated contractility during reperfusion through altered calcium handling[87, 166, 168]. In two recent studies where the mechanism of PKG-mediated protection in PostC was studied, it was shown that the beneficial effects of PKG result from i) delayed normalization of intracellular pH during reperfusion through altered NHE function[228] and ii) antioxidant effects that prevent eNOS uncoupling[91]. Since cGMP has been shown to contribute to the biological effects of H_2S[40, 398], we evaluated the role of cGMP/PKG pathways in H_2S-induced pharmacological conditioning. We observed that DT2 blocked the infarct-limiting effect of NaHS in both rabbits and mice. In line with this finding, we noted that cGMP levels and VASP phosphorylation were increased in the ischemic myocardium.

K_{ATP} channel opening is known to mediate many of the biological activities of H_2S[399]. Given the key role of these channels in pharmacological conditioning mediated by cGMP/PKG, we evaluated their contribution in H_2S-induced cardioprotection, using two different inhibitors, namely 5-HD and glibenclamide. These agents target distinct populations of K_{ATP} channels, as 5-HD inhibits mitoK_{ATP}, while glibenclamide blocks K_{ATP} channels irrespectively of their subcellular localization inhibiting both sarcK_{ATP} and mitoK_{ATP}. *Ex vivo* studies on the role of mitoK_{ATP} vs sarcK_{ATP} have been inconclusive, with some investigators claiming mitoK_{ATP}, sarcK_{ATP} or both types of channels mediate the protective effects of H_2S[249-251, 386] depending on the preparation and conditions used. We found that 5-HD did not limit the beneficial effects of NaHS indicating that mitoK_{ATP} channels do not mediate H_2S-induced cardioprotection *in vivo* in rabbits. Interestingly, administration of glibenclamide *in vivo* reduced the infarct limiting effects of NaHS, in line with the *ex vivo* observations[249-251, 386]. Our data taken together indicate that sarcK_{ATP} channel activation participates in the cardioprotective signaling of H_2S. Sarcolemmal K_{ATP} channels were recently shown to mediate cGMP-dependent natriuretic peptide-induced cardioprotection[87]. Sarcolemmal K_{ATP} channels are involved in intracellular Ca^{2+} handling and their opening results in resistance to apoptosis during I/R injury[400].

We next focused on the possible role of Ca^{2+}-handling proteins in H_2S pharmacological conditioning. We decided to systematically investigate the contribution of PLN, as this protein is a PKG substrate[87, 169, 170]. Moreover, a number of publications have shown altered PLN phosphorylation in the context of I/R[168, 171-173]. In a recent study Inserte et al., showed that phosphorylation of PLN (ser16/thr17) peaked in the control group 3 min after the onset of reperfusion and declined thereafter. On the other hand, following PostC increased PLN phosphorylation was evident in the 5th min of reperfusion. It was, thus, proposed that delayed PLN phosphorylation limits reperfusion-triggered Ca^{2+} oscillations leading to protection[168]. PLN once phosphorylated by PKG reduces free intracellular Ca^{2+} concentration by dissociating from SERCA; SERCA is then able to pump Ca^{2+} ions back into the sarcoplasmic reticulum (SR)[174]. Since no pharmacological inhibitors of PLN exist, the only way to

determine the functional relevance of PLN in PostC or pharmacological conditioning would be through the use of PLN KO. Ablation of the PLN gene in mice is associated with greatly enhanced cardiac Ca^{2+}-cycling and performance. The Ca^{2+} affinity (KCa) of SERCA2a and the overall sensitivity of this transport system for Ca^{2+} is increased. Ca^{2+} uptake rates are higher in PLN KO hearts than WT, especially at low [Ca^{2+}], whereas there is no effect on Vmax[401]. Intriguingly, this hyperdynamic cardiac function is maintained throughout the lifetime of the mouse without observable pathological consequences. The body, heart, and heart/body weight, along with heart rate, mean aortic pressure, cardiac output (venous return), stroke volume and cardiac power (left ventricle) is unaltered compared to wild type mice. Significant changes are however, observed in the left intraventricular pressure (systolic diastolic and end-diastolic), as well as the cardiac contraction and relaxation properties (decreased time to peak pressure, increased absolute values of +dP/dt, decreased half-relaxation time)[401]. In addition to the direct effect of the PLN ablation on SERCA2a, various other compensatory changes in protein expression and/or phosphorylation take place, as demonstrated by global proteome studies, affecting mostly structural and energy related proteins[402]. All of the above should be kept in mind when interpreting data obtained with the PLN KO animals.

To study the role of PLN in H_2S pharmacologic conditioning, we measured PLN phosphorylation in NaHS-treated animals. We observed that PLN was phosphorylated on Ser16/Thr17 after NaHS treatment in rabbits in a PKG-I-dependent manner. Since we only examined a single time-point, we can't comment on the kinetics of phosphorylation during reperfusion or PostC. To determine the contribution of PLN in H_2S-induced cardioprotection, we used PLN KO mice. We found that NaHS failed to restrict infarct size in PLN KO mice after I/R, suggesting that a PKG/PLN pathway mediates the H_2S response. Moreover, our experiments with PLN KO mice indicate that inhibition of PLN abolished the effects of a commonly used PostC algorithm. Since it has been described that during ischemic preconditioning, it is possible to overcome the lost protection of eNOS KO by increasing the number of conditioning cycles[68], we tested a second algorithm of PostC in PLN KO mice. However, increasing the number of cycles from 3 to 6 in the PostC although it showed a trend to limit infarct size, this did not reach statistical significance. Taken together, our results indicate that in addition to its established role in heart failure[174], PLN plays an important role in I/R injury. Although the mechanism of action of PLN in the context of cardioprotection was not studied in detail in the course of our studies, relieving the inhibition on SERCA-mediated Ca^{2+} uptake into the sarcoplasmic reticulum following PLN phosphorylation would be expected to lower free cytosolic Ca^{2+} levels and, thus, limit mitochondrial Ca^{2+} entry[403] and preventing mPTP opening and hypercontracture. Indeed, H_2S has been reported to accelerate sarcoplasmic reticulum-Ca^{2+} uptake rate in single ventricular myocytes[404].

Erk1/2 has been shown to exert a protective role in conditioning. In line with this observation, we found that in rabbits treated with NaHS, Erk1/2 phosphorylation was increased. Erk1/2 activation following exposure to H_2S has been noted before in endothelial cells[405]. Since DT2 treatment reduced Erk1/2 phosphorylation in the rabbit heart under NaHS treatment, Erk1/2 must lie downstream of PKG in the H_2S signaling pathway. Activated Erk1/2 can activate downstream protective pathways to limit infarct size. One of the downstream targets of Erk1/2 is GSK-3β, which once phosphorylated inhibits mPTP opening[406]. In our study, no

evidence for GSK-3β phosphorylation was observed on in H_2S-treated animals, suggesting that Ekr1/2 acts independently of GSK-3β. In contrast, the beneficial effect of NaHS in limiting I/R injury was GSK-3β-mediated in pigs[38]. Divergent results have been noted in experiments in pigs versus rodents have been noted before[187, 407]. The targets of Erk1/2 in the context of H_2S cardioprotection remain to be elucidated. Since PostC utilizes additional pathways to those triggered by NaHS (such as GSK3β and mKATP), we tested whether application of PostC and administration of NaHS have additive effects. In these experiments we observed that the combination of treatments did not confer any benefits over NaHS administration alone. This finding indicates that although PostC and H_2S utilize partially overlapping signaling pathways they likely target a common end effector.

Several lines of evidence suggest that H_2S requires eNOS to exert its protective effects in the cardiovascular system. For example, pharmacological inhibition or genetic ablation of eNOS has been shown to reverse the beneficial effects of H_2S in vasorelaxation[40], angiogenesis[40], wound-healing[40] and resuscitation[408]. In the heart, H_2S donor administration in the context of I/R[33, 396] or heart failure[385, 409], results in increased eNOS phosphorylation and enhanced NO bioavailability. In the most recent study published on H_2S in I/R[33], H_2S donors administered at the end of ischemia resulted in no significant change in infarct size in eNOS KO and eNOS S1179A mice. However, in our initial studies, we found no biochemical or functional evidence for eNOS involvement in the H_2S cardioprotection in rabbits. Given that H_2S is being tested as a cardioprotective agent in human (NCT01989208), we studied whether there are species differences in the mode of action of H_2S. We, thus, repeated the I/R injury experiments in mice. In agreement to what others observed in mice[385, 396, 409] we found that eNOS inhibition abolished the effects of NaHS administration. Our data collectively demonstrate that the protective pathway of H_2S in I/R is dependent on NO in mice, but not in rabbits.

In summary, we have shown that the cardioprotective effects of H_2S are mediated through a cGMP/PKG pathway, independently of mitoK_{ATP} channel opening and GSK-3β inhibition. The pathways responsible for increased cGMP levels include eNOS activation and/or PDE inhibition. Depending on i) the expression levels of eNOS vs PDE and ii) the presence and activity of other signaling components (for example, phosphatases that dephosphorylate eNOS on Ser1177 to restrict its activity) the contribution of these two pathways to cardioprotective cGMP signaling will vary. Thus, H_2S might employ eNOS activation, PDE inhibition or both depending on the species investigated, the experimental conditions used and whether interventions are performed in healthy individuals or in the context of disease. Since different pools of cGMP exist in cells, it is possible that cGMP generated through eNOS activation and cGMP derived from PDE inhibition might exhibit subcellular compartmentalization, recruiting different or overlapping downstream signaling pathways to confer cardioprotection. Furthermore, our study highlights the participation of a novel mediator, namely PLN, in the cardioprotective signaling cascade. The present study expands our knowledge on the molecular mechanisms of action of H_2S in pharmacological conditioning and strengthens the rational on which clinical testing and use of H_2S-releasing compounds can be based.

Experimental Study IV:

Exposure to cigarette smoke abrogates the beneficial effect of ischemic postconditioning.

4.1. Abstract

Background: Cigarette smoking is one of the risk factors for coronary artery disease. Although conditioning decreases infarct size in hearts from healthy animals, co-morbidities may render it ineffective.

Aim: We investigated the effects of cigarette smoke (CS) exposure on intracellular myocardial signaling, infarct size after ischemia/reperfusion and the potential interference with ischemic conditioning.

Methods and Results: Exposure of mice to CS increased blood pressure, caused cardiac hypertrophy, up regulated the NOS/sGC/cGMP pathway whilst decreased H2S generating enzymes expression. To test the effect of CS exposure on the endogenous cardioprotective mechanisms, mice were subjected to regional myocardial ischemia and reperfusion with no further intervention or application of preconditioning (PreC) or postconditioning (PostC). Exposure to CS did not increase the infarction compared to the room air (RA)-exposed group. PreC was beneficial for both CS and RA vs non-conditioned animals. PostC was effective only in RA animals, whilst the infarct size-limiting effect was not preserved in the CS group. Differences in oxidative stress markers, H_2S generating enzymes, Akt and eNOS phosphorylation and cGMP levels were observed between RA and CS groups. Exposure to CS does not *per se* increase infarct size. The beneficial effect of ischemic PreC is preserved in mice exposed to CS, as it does not affect the cardioprotective signaling; in contrast to PostC.

Conclusion: PostC fails to protect CS-exposed mice due to impaired activation of the Akt/eNOS/cGMP axis that occurs in parallel to enhanced oxidative stress.

4.2. Aim

Taking into account that eNOS activation and enhanced NO production[360] constitute a major signaling pathway of conditioning and that smoking in patients leads to endothelial dysfunction and reduced NO bioavailability[410], we hypothesized that smoking might limit the protective effects of PreC and/or PostC. Thus, in the present study we evaluated the impact of CS exposure on infarct size after ischemia/reperfusion (I/R) injury with and without conditioning. Translational efforts to successfully apply conditioning to patients, depends on stratification of human cohorts to select individuals that could potentially benefit from mechanical maneuvers. In this context, identification of co-morbidities that exert a negative impact on PreC/PostC cardioprotection is crucial.

Thus, findings from the current animal study are potentially of great clinical interest, as they could help predict whether application of endogenous cardioprotective mechanisms could still be beneficial if applied to human patients who smoke.

The aim of the present study is:

1. To evaluate the effects of CS exposure on phenotypical heart characteristics.
2. To evaluate the effects of CS exposure on molecular basis in the heart. Focus will be done in the enzymes regulating endogenous NO and H_2S levels.
3. To evaluate the effects of CS exposure on myocardial ischemia-reperfusion injury in a in vivo model of myocardial infarction
4. To assess the possible interaction of CS exposure to the conditioning maneuvers
5. To elucidate the underlying signal transduction pathways (i.e. RISK pathway) and oxidative stress markers in the ischemic heart exposed to CS with the simultaneous application of the conditioning mechanisms

4.3. Materials and Methods

4.3.1. Animals.

Animals received proper care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No.85-23, revised 1996). Approval from the Ethical Committee and the veterinary authorities of Attica prefecture was obtained before the study was initiated. Animals received standards laboratory diet. In the present study male mice C57BL/6 8-12 weeks old were used.

4.3.2. Exposure to cigarette smoke.

In the cigarette smoke group, mice were exposed to dilute mainstream cigarette smoke by using an automated smoking machine for 4 weeks. Mice were exposed to 5 cigarettes (3R4F University of Kentucky, USA), 4 times per day with 30 minutes break, as previously described, while the control group was exposed to air[411].

4.3.3. Murine in Vivo Model of Ischemia-Reperfusion Injury

Described in Experimental Study I, section 1.3.12.1.

4.3.4. Experimental protocol

4.3.4.1. First series of experiments.

Mice were divided in two groups. The first RA_0 (n=5) was exposed to room air and the second CS_0 (n=5) was exposed to cigarette smoke for 4 weeks. The next day after the last exposure to cigarette smoke, animals were sacrificed with an intraperitoneal injection of ketamine (200mg/kg) and xylazine (50mg/kg) anaesthetic cocktail (0.02 ml/g) and whole hearts were rapidly excised from mice, snap frozen to -80^0C and kept for biochemical analysis.

4.3.4.2. Second series of experiments.

Animals were subjected to 30 minutes regional ischemia of the myocardium followed by 2 hr of reperfusion with the following interventions: 1) RA group (n=7): Animals exposed to room air with no further intervention; 2) RA PreC group (n=6): Animals exposed to room air and application of 1 cycle of 5 minutes ischemia followed by 5 minutes of reperfusion before sustained ischemia that lasted 30min; 3) RA PostC 3*10 group (n=8): Animals exposed to room air and application of 3 cycles 10 sec ischemia followed by 10 sec reperfusion immediately after sustained ischemia that lasted 30min; 4) RA PostC 6*10 group (n=8): Animals exposed to room air and application of 6 cycles 10 sec ischemia followed by 10 sec reperfusion immediately after sustained ischemia that lasted 30min; 5) CS group (n=7): Animals exposed to cigarette smoke for 4 weeks with no further intervention; 6) CS PreC group (n=6): Animals exposed to cigarette smoke for 4 weeks and application of 1 cycle 5 minutes ischemia followed by 5 minutes of reperfusion before sustained ischemia that lasted

30min; 7) CS PostC 3*10 group (n=7): Animals exposed to cigarette smoke for 4 weeks and application of 3 cycles 10 sec ischemia followed by 10 sec reperfusion immediately after sustained ischemia that lasted 30min; 8) CS PostC 6*10 group (n=6): Animals exposed to cigarette smoke for 4 weeks and application of 6 cycles 10 sec ischemia followed by 10 sec reperfusion immediately after sustained ischemia that lasted 30min. Reperfusion was allowed to occur for 2hr after which tissue we stained with Evans blue and TTC and infarct to risk area ratios (%I/R) were calculated.

4.3.4.3. Third series of experiments.

C57BL/6 mice (n=6/group) were subjected to the interventions detailed above (groups 1, 2, 3, 5, 6, 7 of the second series of experiments) and allowed to proceed to the 10th minute of reperfusion. At this time, tissue samples from the ischemic part of the myocardium were collected for biochemical analysis.

4.3.5. Western Blot Analysis.

Described in Experimental Study I, section 1.3.4.

The following primary antibodies were used in the present study: phospho eNOS (ser1176), eNOS, iNOS, phospho Akt(ser473), Akt, phospho VASP (Ser239), VASP, PDE5, sGCα1, sGCβ1, PKG, beta actin (Cell Signaling Technology, Beverly, MA, USA), CSE(ProteinTech Group, Inc, Rosemont,USA), CBS(Abnova, Germany) and 3MST (Atlas Antibodies AB, Stockholm, Sweden).

4.3.6. Measurement of oxidative stress biomarkers MDA measurement.

Samples from the ischemic part of the myocardium of all groups (n=6/group) of animals underwent ischemia reperfusion injury were pulverized and then homogenized according to the instruction of each method. The MDA concentration was determined spectrophotometrically and expressed as μM (Oxford Biomedical Research Colorimetric Assay for lipid peroxidation) as previously described[412]. Protein concentration was measured by Lowry method in 750nm. Measurements were performed in triplicate. MDA levels were expressed as μmol/mg protein. Tissue protein carbonyls content was determined by using a modification of the Levine technique, as previously described[412].

4.3.7. cGMP determination.

Described in Experimental Study I, section 1.3.11.

4.3.8. Statistical analysis.

All results are presented as mean ± standard error of the mean. Comparisons of numeric variables among the groups were analyzed using Student's t-test or one-way analysis of variance model (ANOVA) with Bonferroni correction and with Tukey post-hoc analysis. A calculated p value of less than 0.05 was considered to be statistically significant.

4.4. Results

4.4.1. Effects of cigarette smoke exposure on physiological and cardiovascular parameters.

Exposure to cigarette smoke (CS) increased heart weight (Fig. 1A) and heart to body weight ratio versus RA exposed animals (Fig. 1B). The overall body weight was maintained unaffected among groups (Fig. 1C). In addition, exposure to cigarette smoking for 4 weeks increased mean arterial blood pressure from 107 mmHg to 187 mmHg in RA- and CS-exposed anesthetized animals, respectively (Fig. 1D). Heart rate immediately after anesthesia and before any surgical procedure was not different between the two groups of animals (Fig. 1E).

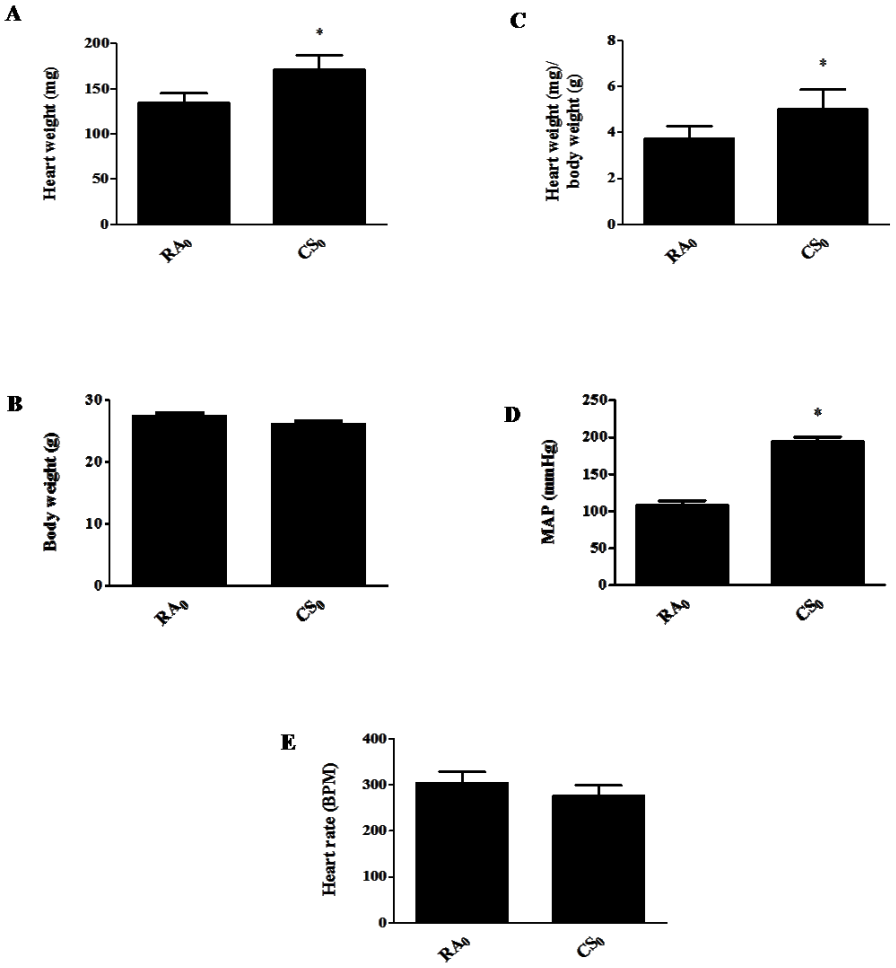


Figure 1: Exposure to cigarette smoke increases heart weight and mean arterial blood pressure. A. Heart weight of C57/BL6 mice 12-16 weeks old. N=18/group, *p<0.05; B. Body weight in g of C57/BL6 mice 12-16 weeks old. n=18/group, p=NS; C. Heart to body weight ratio of C57/BL6 mice 12-16 weeks old. n=18/group, *p<0.05; D. Mean arterial blood pressure in mmHg. n=18/group, *p<0.05 vs RA_0; E. Heart rate in beats per minute. n=18/group, p=NS. CS: Cigarette smoke group; RA: Room Air group

*3.5.2. **Exposure to cigarette smoke alters the expression and activity of the myocardial NO/cGMP pathway.***

Western blot analysis of whole heart lysates from animals exposed to cigarette smoke (CS_o) vs air exposed animals (RA_o) revealed an increase in eNOS (Fig. 2A) and iNOS (Fig. 2B).

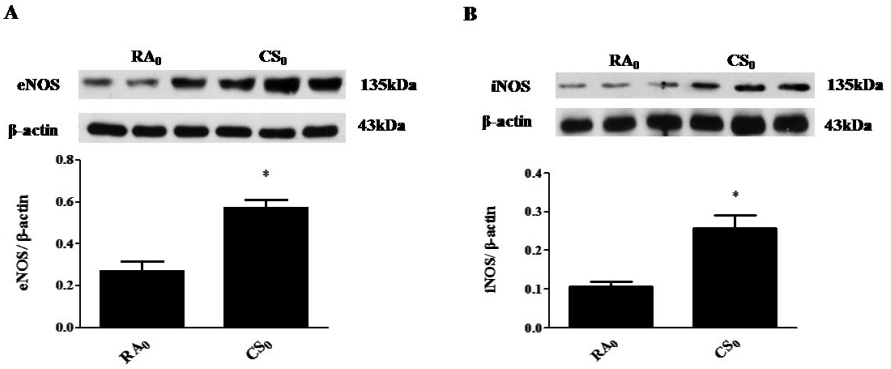


Figure 2: Exposure to cigarette smoke increases NO generating enzymes. Representative western blots and relative densitometric analysis of A. eNOS/β-actin ratio; B. iNOS/β-actin ratio; n=5/group, *$p<0.05$ vs RA_0. CS_0: Cigarette smoke whole heart lysates; RA_0: Room Air whole heart lysates.

Whole heart lysates from animals exposed to cigarette smoke (CS_o) revealed an increase in the protein expression levels of both sGC subunits (Fig. 3A). In contrast, levels of PKG and PDE5 were not affected by cigarette smoking (Fig. 3B&C). In addition, cGMP levels in total heart lysates were higher in the cigarette smoke exposed group (Fig. 3D). To determine whether the elevated cGMP triggered activation of downstream pathways, we measured phosphorylation of VASP on ser239, as readout. Indeed, we observed a statistically significant increase in the levels of phospho-VASP in the hearts of CS mice (Fig. 3E).

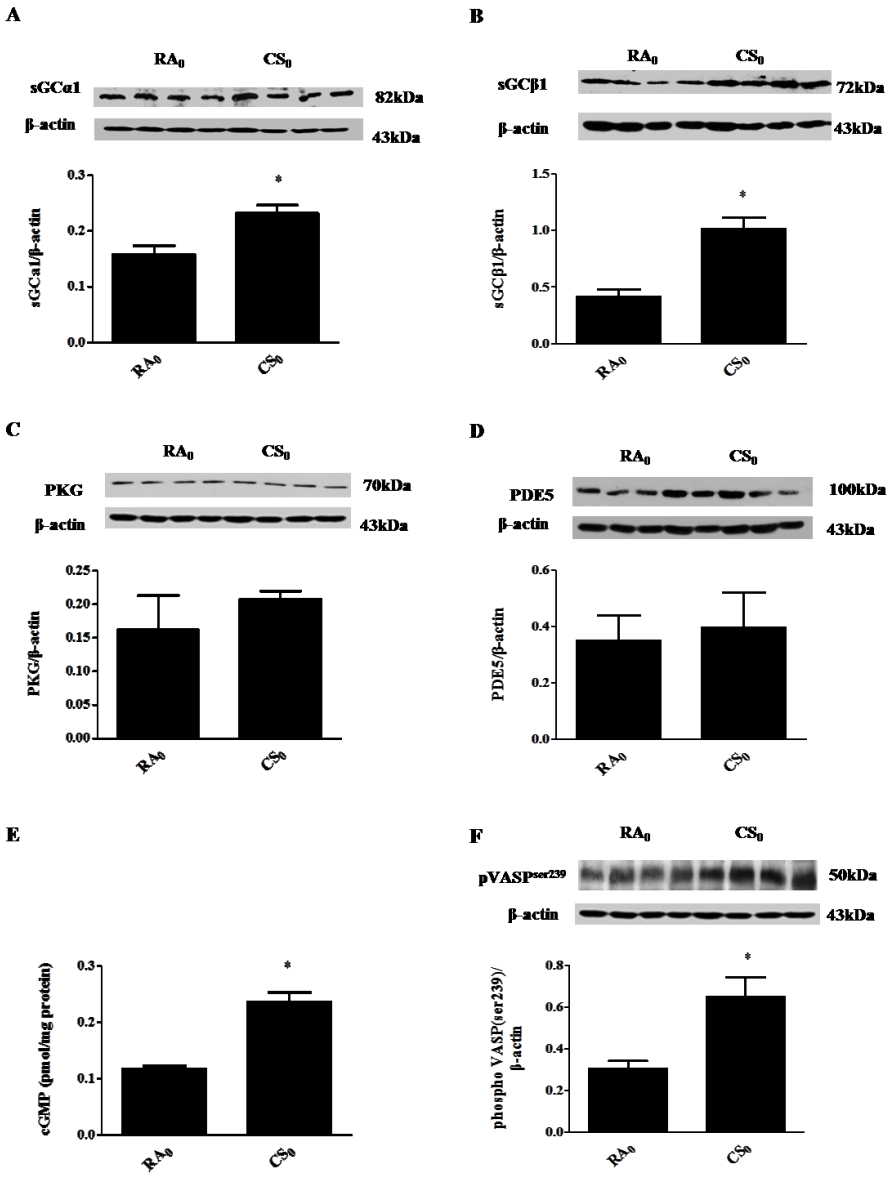


Figure 3: Exposure to cigarette smoke up regulates the sGC/cGMP/PKG pathway. Representative western blots and relative densitometric analysis of A. sGCα1/β-actin; B. sGCβ1/β-actin ratio; C. PKG/β-actin ratio; D. PDE5/β-actin ratio n=5/group, F. pVASP(ser239)/β-actin ratio; and E. cGMP levels in pmol/mg protein, *$p<0.05$ vs RA_0. CS_0: Cigarette smoke whole heart lysates; RA_0: Room Air whole heart lysates.

4.4.3. Exposure to cigarette smoke impairs H_2S generating enzymes.

Western blot analysis in whole heart lysates from animals exposed to cigarette smoke (CS_o) versus air exposed animals (RA_o) revealed a statistical significant decrease on CBS (Figure 4A) and CSE (Figure 4B) protein levels was observed. MPST levels remained unaffected between groups (Figure 4C).

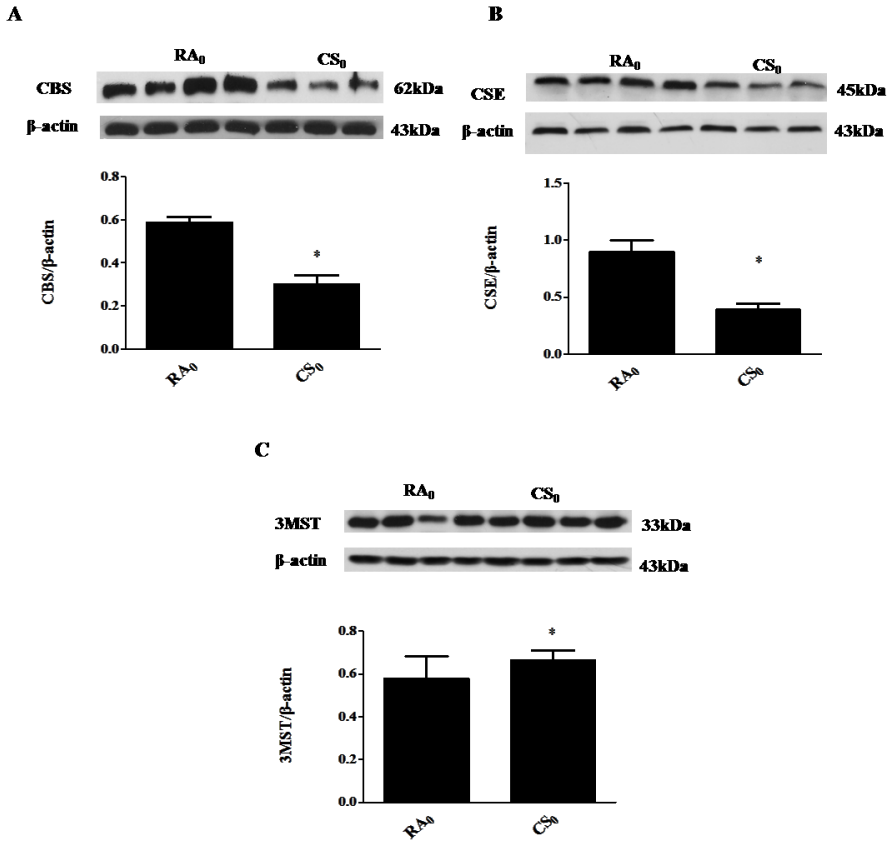
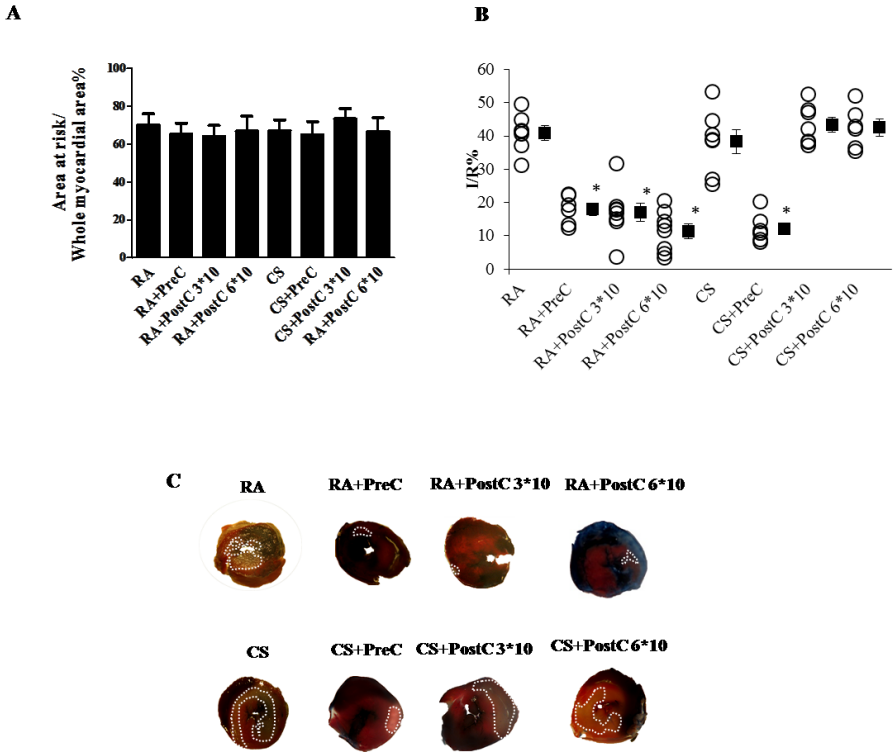


Figure 4: Exposure to cigarette smoke decreases H_2S generating enzymes. Representative western blots and relative densidometric analysis of A. CBS/beta actin ratio, B. CSE/beta actin ratio, C. MPST/beta actin ratio. n=5/group, *$p<0.05$. CS_0: Cigarette smoke; RA_0: Room Air

4.4.4. Exposure to cigarette smoke does not increase infarct size but blunts the cardioprotective effects of postconditioning.

In mice under ischemia/reperfusion no significant differences were detected in the areas at risk among groups (Fig. 5A, 5C). Differences in the infarct size to area at risk ratio (I/R%) are presented in Fig. 5B. Exposure to CS did not increase the infarct size compared to the RA-exposed group. Application of PreC was beneficial for both CS and RA groups. PostC applied in two different algorithms (3 or 6 cycles of 10 sec ischemia followed by 10 sec reperfusion immediately after sustained ischemia), failed to limit infarct size in CS animals, while even the shorter algorithm worked on RA mice.



<u>Figure 5:</u> Exposure to cigarette smoke does not worsen infarct size but abolishes the infarct limiting effects of PostC. A. Area at risk to whole myocardial area % ratio, p=NS; B. Infarcted area to area at risk expressed as % ratio. C. Representative photos from murine hearts of the indicated study groups. *p<0.05 vs RA group or CS control group; CS, Cigarette smoke; RA, Room Air; PreC, Preconditioning; PostC, PostConditioning.

4.4.5. Protection of preconditioning in cigarette smoke exposed animals correlates with activation of Akt/eNOS.

Western blot analysis of ischemic tissue among groups demonstrated that application of PreC and PostC in the RA groups increased activation of Akt, as assessed by Ser473 phosphorylation that led to phosphorylation of eNOS on Ser1176 (Fig. 6A-D).

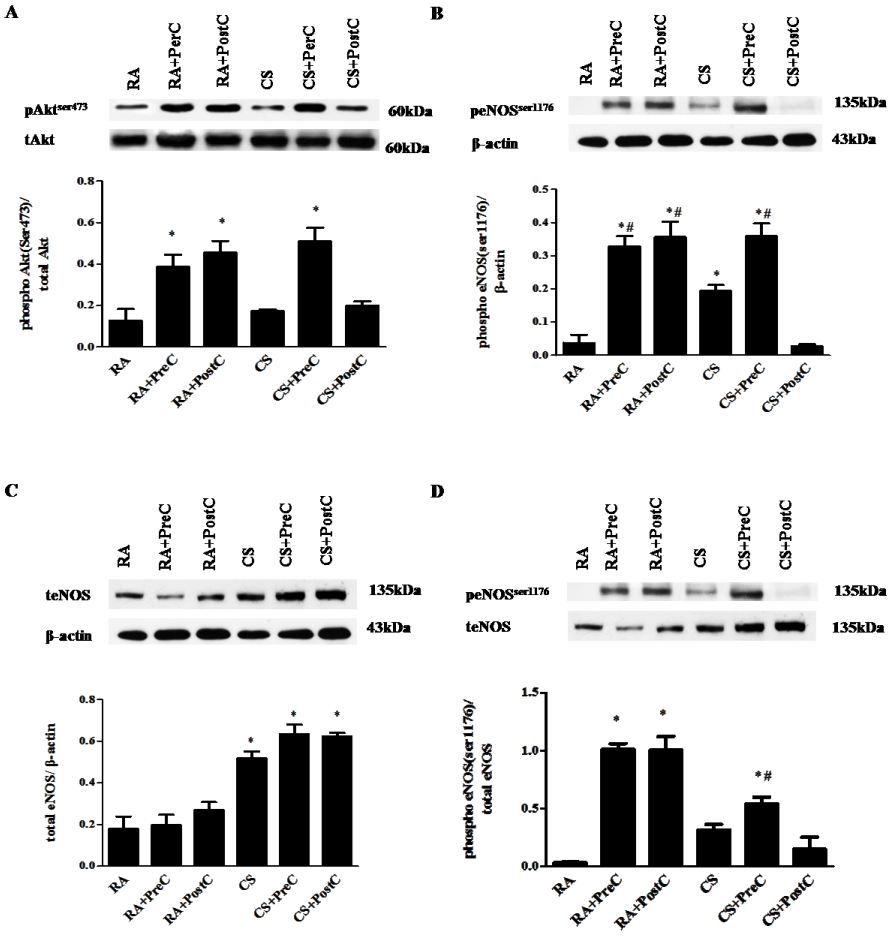


Figure 6: Protection by preconditioning correlates with activation of the Akt/eNOS pathway. Representative western blots and relative densitometric analysis of A. pAkt(ser473)/totalAkt; B. peNOS(ser1176)/β-actin; C. total eNOS ratio/β-actin; and D. peNOS(ser1176)/teNOS ratio. *p<0.05 vs RA group; #p<0.05 vs RA+PreC group; $p<0.05 vs CS group; CS, Cigarette smoke; RA, Room Air; PreC, Preconditioning; PostC, PostConditioning.

In line with these findings, we observed increased cGMP content (Fig. 7A) and phosphoVASP (Fig. 7B) in PreC and PostC RA mice. In control CS mice (without PreC or PostC), Akt phosphorylation remained at levels similar to those of the RA mice. Results in CS animals subjected to mechanical maneuvers varied; PreC increased Akt and eNOS phosphorylation, while PostC enhanced neither (Fig. 7A &D). In addition, PreC in CS mice did not further increase cGMP content and phosphoVASP (Fig. 7A &B). PostC CS mice exhibited reduced cGMP and phospho-VASP levels, compared to CS and CS+PreC groups.

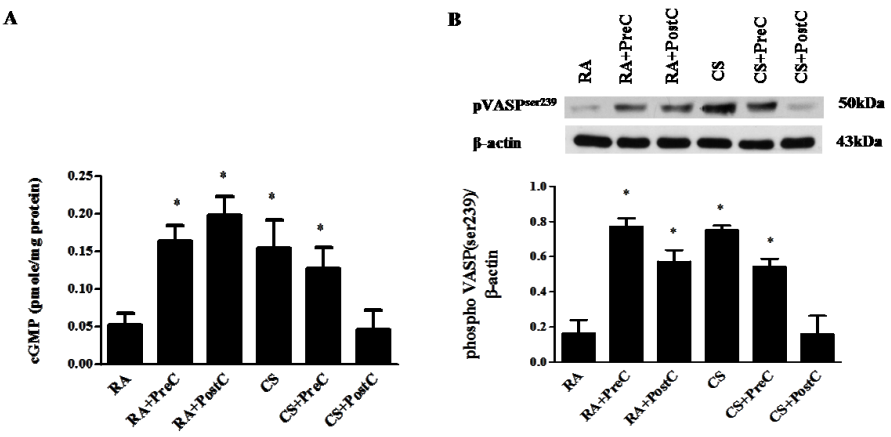


Figure 7: Changes in NO/cGMP signaling after conditioning in cigarette smoking ischemic hearts. A. cGMP levels in the ischemic myocardium in pmol/mg protein and B. representative western blots and relative densitometric analysis of pVASP(ser239)/β-actin ratio. *p<0.05 vs RA. CS, Cigarette smoke; RA, Room Air; PreC, Preconditioning; PostC, PostConditioning.

4.4.6. Lack of protection in cigarette smoke-exposed animals by PostC co-insides with enhanced oxidative stress.

To access the level of oxidative stress we used MDA and PCs as biomarkers. No differences in the levels of MDA and PCs were observed in the ischemic part of the myocardium after 4 weeks of exposure to cigarette smoke. Both PreC and PostC decreased MDA and PCs in RA mice, while only PreC decreased oxidative stress biomarkers in CS mice (Fig. 7A &B).

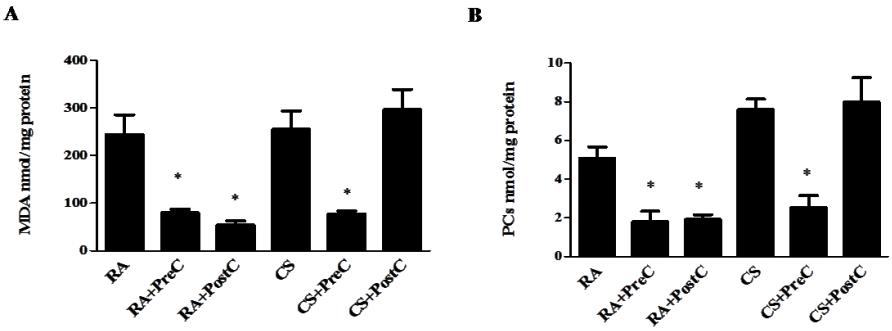


Figure 8: Changes in oxidative stress biomarkers after conditioning in cigarette smoking ischemic hearts. A. MDA in nmol/mg protein; B. PCs in nmol/mg protein. *p<0.05 vs RA; CS, Cigarette smoke; RA, Room Air; PreC, Preconditioning; PostC, PostConditioning; MDA, malondialdehyde; PCs, protein carbonyls

4.5. Discussion

The main findings of the present study are that exposure of mice to CS does not impact on the infarct size observed after I/R and that cardioprotection by PostC, but not PreC, is lost in CS mice. Initially, we sought to characterize basic cardiovascular parameters in mice exposed to CS. We observed that after 4 weeks, mice treated with CS exhibited enhanced mean arterial blood pressure, while showing no difference in body weight and heart rate. Our findings regarding body weight and blood pressure are in line with those of Guo et al[413], who showed increased mean pressure and no change in body weight of mice after 6 weeks of CS exposure. Decreased body weight has been reported to occur in mice exposed to cigarette smoke, but takes longer to develop[297].

Acute (but not chronic) CS exposure has been found to paradoxically protect the endothelium from ischemia reperfusion injury in patients[414]. In our study, exposure of mice to 4 weeks of CS did not affect the infarct size after LAD ligation and reperfusion. In contrast, Zhu et al. reported that exposure to environmental tobacco smoke for 6 weeks increased myocardial infarct size in a rat model of ischemia/reperfusion[296]. The reasons for these conflicting results could be due to the use of non-standardized cigarettes[296] or might reflect species differences. To further study the changes induced by smoking in heart tissue, we measured the levels of enzymes in the NO/cGMP pathway and found that in mice exposed to CS, eNOS, iNOS and sGC levels were increased in the heart, raising the possibility that these molecular changes in the smoke-exposed myocardium could serve as a compensatory mechanism. eNOS levels have been shown to be reduced in vessels from mice exposed to CS[413], while increased eNOS expression was observed in endothelial cells exposed to plasma from smokers[415]. Generation of NO from eNOS depends not only on the absolute levels of the enzyme, but also on BH_4 availability and eNOS coupling, as well as posttranslational modifications of eNOS[416, 417]. Exposure to cigarette smoke or cigarette extracts leads to dissociation of the active dimeric eNOS, eNOS acetylation and phosphorylation of eNOS on the inhibitory site Thr495[275, 418], all of which are consistent with reduced eNOS activity. We observed that eNOS in addition to being present at higher levels in the myocardium, is also hyperphosphorylated on Ser1176, a site linked to enhanced NO production. While eNOS activity was not directly measured in our experiments, the increase in cGMP content and VASP phosphorylation in myocardial tissue of CS-exposed mice suggest that more biologically active NO is produced in the hearts of these mice. Obviously, the increase in cGMP could also result from the higher amounts of sGC present in CS hearts, in addition to the enhanced levels of iNOS. Interestingly, iNOS has been shown before to be protective in the heart in the context of ischemia/reperfusion injury, in contrast to what is observed in other organs[83]. All of the above taken together suggest that the NO/cGMP pathway is more active in CS vs RA-exposed mice.

In addition, except NO, hydrogen sulfide exerts similar properties and both endogenous mediators are necessary for the cardiovascular function under physiological conditions[43, 385, 399, 419]. H_2S therapy has been proposed to improve left ventricular remodeling in smoking rats by exerting its anti-oxidant effects[420]. In the present study, we found a decrease in CBS and CSE protein levels; the decrease in the protein levels of CBS and CSE may reflect a lower level of H_2S in the cardiovascular system. This could easily explain the increased blood pressure, as H_2S is a potent vasodilator[399] and the impairment of its levels is observed in hypertension[421].

We next determined whether the cardioprotective effects of PreC and PostC are altered in animals exposed to CS. When mice exposed to RA were subjected to PreC and PostC, infarct size was significantly reduced. In contrast, CS-exposed mice were only protected by PreC. PostC by three cycles of 10 s/10 s ischemia/reperfusion in aged mice failed to reduce the infarct size. However, changing the postconditioning algorithm and increasing the number of conditioning cycles in aged mice was shown to restore protection[191]. In our study, we observed that increasing the number of PostC cycles in CS mice did not reduce infarct size. Similarly to what was observed in CS-exposed mice, cardioprotection by PostC is lost in a number of disease conditions and co-morbidities, such as left ventricular dysfunction, hypercholesterolemia, diabetes or aging[117]. However, there are divergent results in the literature regarding the effect of PostC in hypertension. PostC exerts cardioprotection in the presence of clearly established hypertrophy, as well as in conditions in which hemodynamic overload progresses toward systolic dysfunction[276]; PostC decreased myocardial dysfunction in spontaneously hypertensive rats (SHR)[422]. In contrast, it was reported that PostC neither improved contractility, nor reduced infarct size in SHR hearts[274, 423]. All of the above-mentioned studies were performed in isolated hearts; in the only available *in vivo* study, myocardial hypertrophy in SHR was proposed to abrogate the effect of PostC[424]. Herein, we demonstrated that smoking resulted in hypertension and cardiac hypertrophy, abolishing the protective effect of PostC. The reason why only one of the two endogenous mechanisms of cardioprotection (PostC) becomes ineffective in the above-mentioned conditions, especially since PreC and PostC share common molecular pathways[425], is not known.

We next investigated the mechanism(s) that mediates cardioprotection and are affected by CS, leading to loss of protection in PostC, focusing on the eNOS/cGMP pathway. Initially, we measured Akt phosphorylation as an index of Akt activity[161], since this kinase is a major regulator of eNOS activity in the cardiovascular system. Akt phosphorylation was low in the control RA ischemic myocardium and was activated in the RA animals, in which pre and postconditioning were applied, an observation which is in line with the literature[426]. In the CS-exposed ischemic myocardium, Akt was activated only in the CS+PreC group, whereas no activation was observed in animals subjected to PostC. In agreement to the observed up regulation in Akt activity, eNOS phosphorylation was increased in the myocardium following conditioning in RA-exposed animals. However, the peNOS/eNOS ratio was found to be enhanced in non-conditioned CS mice in the absence of enhanced Akt phosphorylation and only increased in the CS+PreC group, while in CS+PostC the ratio tended to be lower than that observed in non-conditioned animals. These findings in CS-exposed mice are in accordance with our observation that PostC does not enhance phosphorylation of eNOS and Akt in the ischemic region of myocardium of hypercholesterolaemic animals[248].

Following myocardial reperfusion upon opening of the occluded LAD, the electron transport chain gives rise to ROS; ROS are also generated through xanthine oxidase and NADPH oxidase[18]. ROS mediate myocardial reperfusion injury by opening of the mPTP, acting as chemoattractant for neutrophils, and impacting on the sarcoplasmic reticulum function[427]. ROS also cause lipid peroxidation damaging the cell membrane and inhibit enzyme function. As far as the NO pathway is concerned, increased generation of superoxide anions reduces the bioavailability of NO, converting it to peroxynitrite and leads to oxidation of BH_4, a cofactor required for NOS coupling that is depleted during ischemia/reperfusion[418]. To determine the

level of oxidative stress in RA and CS mice, we measured PCs and MDA as biomarkers in the ischemic part of the myocardium, in animals subjected to PreC and PostC[248]. No differences in the baseline values of PC and MDA were observed between RA and CS mice; it should be noted that both biomarkers were determined 24hr after the last exposure to CS and that although no differences we observed in the heart, lung PC and MDA are elevated in CS mice (data not shown). In line with what is known from the literature regarding the reduction of deleterious ROS burst by conditioning[428], both PC and MDA were reduced in RA exposed mice after conditioning. However, although PreC limited oxidative stress biomarkers, PC and MDA remained high in PostC CS-exposed animals, paralleling the loss of cardioprotection in the CS+PostC group; this observation is once again similar to what we observed in hypercholecterolemic rabbits[248].

Although administration of cGMP-elevating agents mimics the effect of conditioning in ischemia/reperfusion injury[166], the role of S-nitros(yl)ation vs cGMP-dependent pathways in PreC and PostC is still unresolved[163, 166]. Moreover, no data on the role of cGMP on infarct size in animals with co-morbidities have been reported. In our experiments, GMP levels and VASP phopshorylation were found to be increased after PreC and PostC in RA animals. cGMP levels were also high in the CS and CS+PreC groups, but reduced in the CS+PostC group. The reason why cGMP levels were even lower in the CS+PostC compared to the CS group is unknown, but suggests a lower synthesis or increased degradation of this cyclic nucleotide. Recent studies have proposed that the beneficial effects of cGMP in PostC are independent of the PI3K/Akt pathway, but are rather due to enhanced activation of eNOS that results from reduced superoxide anion production at the onset of reperfusion, leading to attenuated oxidation of BH_4 and reduced NOS uncoupling[91]. Thus, the inability of PostC to attenuate oxidative stress in the CS group might explain the lack of cardioprotection and the decreased cGMP levels that were observed in CS+PostC group.

Results from the present study are potentially of great translational interest, since although PostC is a clinically relevant and powerful mechanism of reducing ischemia/reperfusion injury in humans, clinical trials have been largely disappointing [reviewed in[429]]. Smoking and hypertension are two major risk factors in the development of systemic atherosclerosis and coronary artery disease, acting either as independent or as additive predisposing factors for CVD[430]. It was recently demonstrated that the efficacy of remote ischemic conditioning is reduced in smokers, whereas hypertensive patients, experienced protection by remote conditioning[431]. In the present study we used an animal model of CS exposure that lead to hypertension development, rather than a model of spontaneous hypertension. The model used in our study is analogous to smokers who develop hypertension and left ventricular hypertrophy and if our observations can be extrapolated to humans, this patient population would be protected by PreC, but not PostC. This would be in agreement with the many preclinical studies which have reported differences in the effectiveness of preconditioning and postconditioning in the presence of co-morbidities[117]. Further studies are required to elucidate the exact molecular mechanism(s) responsible for limiting the effectiveness of PostC in smokers and to target them so that cardioprotection is restored.

General Discussion and Future Perspectives

General Discussion

Acute myocardial infarction (AMI) is a serious and often fatal consequence of coronary artery disease (CAD). An imbalance in oxygen/nutrient supply and demand within the ischemic organ results in tissue hypoxia and dysfunction. Subsequent reperfusion in addition to its beneficial effects can enhance innate responses that lead to cell death programs. Despite the knowledge gathered over 30 years of experimentation, no pharmacological strategies are applicable that could rescue the heart muscle in patients with myocardial infarction after reperfusion. Diverse therapeutic attempts, have all failed so far to enter everyday clinical practice, in spite of positive proof-of-concept studies in experimental animals. Recent advances in understanding the molecular events associated with ischemia and reperfusion may lead to innovative therapeutic strategies for patients with ischemia-reperfusion injury–associated tissue and organ dysfunction.

In this context in the **Experimental Study I**, we sought to evalutate deeper the role of a novel kinase PYK2 in the post-translational modifications (i.e. phosphorylation) of eNOS in myocardial ischemia-reperfusion injury. Endothelial nitric oxide synthase (eNOS) is constitutively expressed in cardiomyocytes and endothelial cells. eNOS is phosphorylated on serine, threonine and tyrosine residues and this alters the enzyme's ability to produce NO. Undoubtedly, phosphorylation of S1177 due to its prominent role in regulating eNOS activity has attracted the most attention, with many studies, mistakenly, equating phosphorylation on this residue with proof of enhanced eNOS activity in the heart. Another site commonly studied is T495, that is located in the calmodulin (CaM)-binding domain and exerts a negative effect on eNOS activity. More recently Y657 was reported to be a critical determinant of its enzymatic activity; phosphorylation of Y657 (mouse Y656) by proline-rich tyrosine kinase 2 (PYK 2) exposed to fluid shear stress or insulin inhibits eNOS activity. PYK2, a kinase that negatively regulates eNOS activity, is activated by elevations in calcium and by oxidative stress, both of which hallmarks of I/R injury. Although PYK2 has been linked to adverse cardiac phenotypic changes, such as hypertrophy and lethal arrhythmias, its role in ischemia-reperfusion injury remains unexplored. In the present study, by using in vitro and in vivo models of ischemia-reperfusion injury we set out to determine whether PYK2 is activated during ischemia-reperfusion injury, affecting the ability of eNOS to confer cardioprotection. Our major finding is that PYK2-driven phosphorylation of eNOS on Y656 promotes cardiomyocyte death and increases infarct size. Thus, PYK2 emerges as a novel therapeutic target that restores eNOS signaling and function in ischemia-reperfusion injury and minimizes cardiac necrosis.

However, to solve the unsolved problem of myocardial ischemia-reperfusion injury and to improve clinical outcome and prevention of the lethal reperfusion injury, an intervention is needed that would make the heart muscle resistant to necrosis. During the last two decades, scientific efforts focus on improving means for limiting infarct size. However, the morbidity and mortality of CAD remain significant worldwide and lay the ground for the development of novel cardioprotective therapies.

Ischemic conditioning is the protective mechanism, by which brief ischemia renders the heart resistant to potentially lethal ischemia and reperfusion. The two main mechanisms of conditioning are ischemic preconditioning (PreC) and ischemic postconditioning (PostC), both preventing the development of necrosis due to reperfusion injury. Although PreC is a powerful form of protection, it is of limited clinical application (i.e. transplantation) as it is

applied before the ischemic. On the other hand PostC is applicable at the time of reperfusion, making it a more attractive strategy for use in the clinical routine.

Upon their mechanical application intracellular signal transduction takes place via at least three parallel pathways; the first two are activated by receptors coupled with G proteins (GPCR) and proceeds via a nitric oxide (NO), cGMP, and PKG (protein kinase G) pathway or activation of reperfusion injury salvage kinases pathway (RISK pathway, which includes PI3K, Akt, ERK and GSK3beta). The third, which is activated by TNF-alpha, includes mitochondrial transactivation of transcription molecules (SAFE pathway). All three pathways rely on preservation of the mitochondrial function by preventing the mPTP opening resulting in rendering the ischemic heart lee vulnerable to reperfusion injury.

Despite the huge interest and the great body of evidence that verify the effectiveness of postconditioning, clinical application has remained limited due to many factors that may enhance or blur its protective effect. Since the therapeutic benefit of mechanical postconditioning has been challenged, administration of pharmacological agents that act as postconditioning mimetics might be a preferred approach. The use of a safe drug as a postconditioning agent is fairly easy, if it is provided in an intravenous formulation and can be administered in a manner that cover the time window for protection against reperfusion injury.

In this context of pharmacological conditioning a novel concept of "gasotransmitter" arrived recently. Gasotransmitters are small molecules of endogenous gases with important physiological functions. Their production and metabolism are enzymatically regulated, and their effects are not dependent on specific membrane receptors. Among them NO and H_2S has shown remarkable effects on the (patho) physiology of heart muscle and ischemia reperfusion injury.

In this context in **Experimental Studies II** and **III**, we sought to evaluate the possible cardioprotective actions of exogenous administrated NTG (a potent NO donor) and NaHS (a rapid releasing H_2S donor) on myocardial infarct size. In turn we evaluated the underlying molecular mechanisms involved. Our major findings were that both gasotransmitters administration during the clinical setting of the ischemic insult minimize myocardial infarct size. However, they differ in the underlying mechanism. On one hand NO donor, NTG reduces myocardial infarction in a PI3K-eNOS pathway, which further reduces oxidative stress and inhibits mPTP opening, independent of PKG activation in the ischemic heart. In addition, NTG showed a remarkable effect on infarct size of a model of endothelial dysfunction. On the other hand, H_2S donor, NaHS, exerts its beneficial effects in a cGMP/PKG/PLN dependent pathway, by inhibiting PDE activity. Although, distinct mechanisms of action are described in these experimental studies, donors that recapitulate the endogenous produced gasotransmitters may serve as possible pharmacological treatment during myocardial infarction to improve cell viability. Thus we suggest i) that by titration of the infusion rate of a widely used NO releasing drug NTG, elevated left ventricular filling pressure might fell to within normal limits and work in favor of infarct size limitation even in humans with endothelial dysfunction. And ii) that we expanded our knowledge on the molecular mechanisms of action of H_2S in pharmacological conditioning and strengthens the rational on which clinical testing and use of H_2S-releasing compounds can be based.

However, although great efforts have been made in the understudying of i) pathophysiology of ischemia-reperfusion injury; ii) underlying mechanisms of the application of conditioning maneuvers and iii) novel pharmacological conditioning strategies, the findings cannot be

translated in clinical practice to improve human outcomes following acute myocardial infarction. The reasons are more than one. First, many of the trials performed over the last two decades were small, single-center studies, frequently with few participants and a short follow-up period. As such, they have lacked the power needed to investigate the hard end points of death or a major cardiovascular event. Second, the effectiveness of ischemic-conditioning strategies in humans seems to be less profound than reported in the animal literature, with some randomized clinical trials showing no significant benefit. A possible explanation for this is the effect of underlying comorbidities on the ability of tissues to respond to the beneficial effects of ischemic conditioning. When extrapolating from animal models to humans it is vital to understand the differences between animal models and patients.

In this context, we performed the **Experimental Study IV**, in order to evaluate the capability of conditioning mechanisms to limit infarct size during a new described comorbidity-cigarette smoke exposure. Inhalation of smoke ingredients and direct nicotine exposure during cigarette smoking cause constriction of epicardial and resistance vessels, impair endothelium-dependent dilatation and deteriorate the elastic properties of vessels in humans. All these unfavorable effects are involved in the development of atherosclerosis, arterial hypertension and left ventricular hypertrophy. To date, there are no published studies designed to investigate the effects of exposure to CS and its sequelae on conditioning. Our major findings are that exposure of mice to CS does not impact on the infarct size observed after ischemia reperfusion injury and that cardioprotection by PostC, but not PreC, is lost in CS mice. A compensatory mechanism of heart exposed to CS has been revealed including increased cGMP/PKG signaling. PreC manages to minimize infarct size in this model by preserving the compensatory mechanism and reducing oxidative stress in the smoke exposed ischemic heart, whilst PostC fails to do so. Thus, findings from the current animal study are potentially of great clinical interest, as they could help predict whether application of endogenous cardioprotective mechanisms could still be beneficial if applied to human patients who smoke.

Future Perspectives

The field of myocardial infarction has reached a plateau as although the interesting findings in the animal models no safe drug or intervention has become clinical routine. In this context the physiology of gasotransmitters -NO and H_2S- both endogenously produced or exogenously administrated are promising agents for the treatment of heart ischemia.

However outstanding questions that need to be assessed and further evaluation of novel mechanisms have to be evaluated:

1. The first question arising is what is the physiological concentrations of NO and H_2S in the heart muscle during the ischemia-reperfusion injury. Do their levels change? In this context novel methods on evaluating with accuracy the circulating or tissue levels of these gaseous molecules should be designed. This could serve as development of possible circulating markers indicative of cell death or novel insights on therapeutic drugs which could equilibrate NO and H_2S levels during the injury.
2. The enzymatic activity of both their key generating enzymes should be assessed. Especially assessment of CSE activity-as the basic H_2S generating enzyme in the

cardiovascular system- is mandatory. Further studies on post-translation modifications (i.e. phosphorylation) of the CSE enzyme will prove its regulation during myocardial infarction.

3. Novel insights on the epigenetic changes (i.e. non-coding RNA regulation) during myocardial ischemia reperfusion has been recently described. A better link between these novel mechanisms of myocardial cell death to the pathophysiology of ischemia-reperfusion injury, conditioning mechanisms, and gasotransmitters mode of action could shed more light on the unsolved problem of myocardial infarction therapy.

4. Finally, meta-analysis studies in humans are necessary to evaluate the stoppers of cardioprotective strategies in humans. This could lead in the better design of experimental studies. The role of gasotransmitters in co-morbidities is still in its infancy. More studies on circulating levels, enzymatic activities and regulation, treatment with exogenously administrated drugs and use of suitable knockout models are needed to finally interpret ate the results in patients.

Abbreviations

3MST; 3-mercaptopyruvate sulfurtransferase

Akt; protein kinase B

AR; adenosine receptor

Atg; Autophagy Related Protein

ATP; adenosine triphosphate

Bak; bcl-2 homologous antagonist-killer protein

Bax; BCL2-Associated X Protein

Bcl2; B-cell lymphoma 2 protein

BH4; tetrahydrobiopterin

CBS; cystathionine beta synthase

cGMP; Cyclic guanosine monophosphate

CGRP; calcitonin gene-related peptide

CHD; coronary heart disease

CS; cigarette smoke

CSE; cystathionine gamma lyase

DHE; dihidroethidium

ECG; electrocardiogram

eNOS; endothelial nitric oxide synthase

EPR; Electron paramagnetic resonance

ERK; Extracellular signal-regulated kinase

FAD; flavin adeninde dinucleotide

FADD; Fas-Associated protein with Death Domain

FMN; flavin mononucleotide

GSK; glycogen synthase kinase

HRP; horshradise peroxidase

HSP; heat shock protein

iNOS; inducible nitric oxide synthase

LAD; left anterior descending

LDH; lactate dehydrogenase

L-NAME; L-NG-Nitroarginine methyl ester

LVH; left ventricular hypertrophy

MMP; matrix metalloproteinase

mPTP; mitochondrial permeability transition pore

mTOR; mammalian target of rapamycin

MTT; (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium

NADPH; Nicotinamide adenine dinucleotide phosphate

NHE; Na^+/H^+ Exchange

nNOS; neuronal nitric oxide synthase

NOHA; NN-hydroxy-L-arginine

NOS; nitric oxide synthase

PAG; DL-Propargylglycine

PCI; percutaneous coronary intervention

PI3K; Phosphoinositide 3-kinase

PKC; protein kinase C

PKG; protein kinase G

PLN; phospholamban

PostC; ischemic postconditioning

PreC; ischemic preconditioning

PYK2; proline rich tyrosine kinase 2

RIP; receptor interacting protein

RISK; reperfusion injury salvage kinase

ROS; reactive oxygen species

S1P; sphingosine-1-phosphate

SAFE; survivor activating factor enchancement pathway

SERCA; sarcoplasmic reticulum calcium transport ATPase

Smac/DIABLO; Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein

SNAP; S-Nitroso-N-Acetyl-D,L-Penicillamine

SR; sarcoplasmic reticulum

STEMI; ST-segment elevation myocardial infarction

TNF; tumor necrosis factor

TRADD; Tumor necrosis factor receptor type 1-associated DEATH domain

TRAIL; TNF-related apoptosis-inducing ligand

TTC; triphenyltetrazolium

VASP; Vasodilator-stimulated phosphoprotein

VDAC; voltage-dependent anion channel

References

1. Guidlines AHA. *p14*. 2015

2. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G. Classification of cell death: Recommendations of the nomenclature committee on cell death 2009. *Cell Death Differ*. 2009;16:3-11

3. Moquin D, Chan FK. The molecular regulation of programmed necrotic cell injury. *Trends Biochem Sci*. 2010;35:434-441

4. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev*. 2007;87:99-163

5. Whelan RS, Kaplinskiy V, Kitsis RN. Cell death in the pathogenesis of heart disease: Mechanisms and significance. *Annu Rev Physiol*. 2010;72:19-44

6. Botting KJ, Wang KC, Padhee M, McMillen IC, Summers-Pearce B, Rattanatray L, Cutri N, Posterino GS, Brooks DA, Morrison JL. Early origins of heart disease: Low birth weight and determinants of cardiomyocyte endowment. *Clin Exp Pharmacol Physiol*. 2012;39:814-823

7. Gottlieb RA, Mentzer RM. Autophagy during cardiac stress: Joys and frustrations of autophagy. *Annu Rev Physiol*. 2010;72:45-59

8. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet*. 2009;43:67-93

9. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008;132:27-42

10. Kubli DA, Gustafsson AB. Cardiomyocyte health: Adapting to metabolic changes through autophagy. *Trends Endocrinol Metab*. 2014;25:156-164

11. Smith CC, Yellon DM. Necroptosis, necrostatins and tissue injury. *J Cell Mol Med*. 2011;15:1797-1806

12. Vandenabeele P, Declercq W, Van Herreweghe F, Vanden Berghe T. The role of the kinases rip1 and rip3 in tnf-induced necrosis. *Sci Signal*. 2010;3:re4

13. Morgan MJ, Kim YS, Liu ZG. Tnfalpha and reactive oxygen species in necrotic cell death. *Cell Res*. 2008;18:343-349

14. Baines CP. The mitochondrial permeability transition pore and ischemia-reperfusion injury. *Basic Res Cardiol*. 2009;104:181-188

15. Baines CP. The molecular composition of the mitochondrial permeability transition pore. *J Mol Cell Cardiol*. 2009;46:850-857

16. Halestrap AP. What is the mitochondrial permeability transition pore? *J Mol Cell Cardiol*. 2009;46:821-831

17. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med*. 2007;357:1121-1135

18. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: A neglected therapeutic target. *J Clin Invest*. 2013;123:92-100

19. Avkiran M, Marber MS. Na(+)/h(+) exchange inhibitors for cardioprotective therapy: Progress, problems and prospects. *J Am Coll Cardiol*. 2002;39:747-753

20. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: Preconditioning, postconditioning, and translational aspects of protective measures. *Am J Physiol Heart Circ Physiol*. 2011;301:H1723-1741

21. Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM. Calcium-mediated cell death during myocardial reperfusion. *Cardiovasc Res*. 2012;94:168-180

22. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol*. 2012;298:229-317

23. Eltzschig HK, Eckle T. Ischemia and reperfusion--from mechanism to translation. *Nat Med*. 2011;17:1391-1401

24. Wang R. Two's company, three's a crowd: Can h2s be the third endogenous gaseous transmitter? *FASEB J*. 2002;16:1792-1798

25. Predmore BL, Lefer DJ, Gojon G. Hydrogen sulfide in biochemistry and medicine. *Antioxid Redox Signal*. 2012;17:119-140

26. Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y, Du J, Tang C. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. *Biochem Biophys Res Commun*. 2004;318:756-763

27. Zhu YZ, Wang ZJ, Ho P, Loke YY, Zhu YC, Huang SH, Tan CS, Whiteman M, Lu J, Moore PK. Hydrogen sulfide and its possible roles in myocardial ischemia in experimental rats. *J Appl Physiol (1985)*. 2007;102:261-268

28. Szabo G, Veres G, Radovits T, Gero D, Modis K, Miesel-Groschel C, Horkay F, Karck M, Szabo C. Cardioprotective effects of hydrogen sulfide. *Nitric Oxide*. 2011;25:201-210

29. Chuah SC, Moore PK, Zhu YZ. S-allylcysteine mediates cardioprotection in an acute myocardial infarction rat model via a hydrogen sulfide-mediated pathway. *Am J Physiol Heart Circ Physiol*. 2007;293:H2693-2701

30. Li C, Guo Z, Guo B, Xie Y, Yang J, Wang A. Inhibition of the endogenous cse/h(2)s system contributes to hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Mol Med Rep*. 2014;9:2467-2472

31. Wang Q, Liu HR, Mu Q, Rose P, Zhu YZ. S-propargyl-cysteine protects both adult rat hearts and neonatal cardiomyocytes from ischemia/hypoxia injury: The contribution of the hydrogen sulfide-mediated pathway. *J Cardiovasc Pharmacol*. 2009;54:139-146

32. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A, Lefer DJ. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. *Circulation*. 2010;122:11-19

33. King AL, Polhemus DJ, Bhushan S, Otsuka H, Kondo K, Nicholson CK, Bradley JM, Islam KN, Calvert JW, Tao YX, Dugas TR, Kelley EE, Elrod JW, Huang PL, Wang R, Lefer DJ. Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc Natl Acad Sci U S A*. 2014;111:3182-3187

34. Sivarajah A, McDonald MC, Thiemermann C. The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. *Shock*. 2006;26:154-161

35. Zhuo Y, Chen PF, Zhang AZ, Zhong H, Chen CQ, Zhu YZ. Cardioprotective effect of hydrogen sulfide in ischemic reperfusion experimental rats and its influence on expression of survivin gene. *Biol Pharm Bull*. 2009;32:1406-1410

36. Sodha NR, Clements RT, Feng J, Liu Y, Bianchi C, Horvath EM, Szabo C, Stahl GL, Sellke FW. Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischemia/reperfusion injury. *J Thorac Cardiovasc Surg*. 2009;138:977-984

37. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabo C, Kimura H, Chow CW, Lefer DJ. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc Natl Acad Sci U S A*. 2007;104:15560-15565

38. Osipov RM, Robich MP, Feng J, Liu Y, Clements RT, Glazer HP, Sodha NR, Szabo C, Bianchi C, Sellke FW. Effect of hydrogen sulfide in a porcine model of myocardial ischemia-reperfusion: Comparison of different administration regimens and characterization of the cellular mechanisms of protection. *J Cardiovasc Pharmacol*. 2009;54:287-297

39. Asimakopoulou A, Panopoulos P, Chasapis CT, Coletta C, Zhou Z, Cirino G, Giannis A, Szabo C, Spyroulias GA, Papapetropoulos A. Selectivity of commonly used pharmacological inhibitors for cystathionine beta synthase (cbs) and cystathionine gamma lyase (cse). *Br J Pharmacol*. 2013;169:922-932

40. Coletta C, Papapetropoulos A, Erdelyi K, Olah G, Modis K, Panopoulos P, Asimakopoulou A, Gero D, Sharina I, Martin E, Szabo C. Hydrogen sulfide and nitric oxide are mutually dependent in the regulation of angiogenesis and endothelium-dependent vasorelaxation. *Proc Natl Acad Sci U S A*. 2012;109:9161-9166

41. Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. *Cardiovasc Res*. 2004;61:402-413

42. Cheng Q, Sedlic F, Pravdic D, Bosnjak ZJ, Kwok WM. Biphasic effect of nitric oxide on the cardiac voltage-dependent anion channel. *FEBS Lett*. 2011;585:328-334

43. Ziolo MT, Kohr MJ, Wang H. Nitric oxide signaling and the regulation of myocardial function. *J Mol Cell Cardiol*. 2008;45:625-632

44. Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. Protein s-nitrosylation: Purview and parameters. *Nat Rev Mol Cell Biol*. 2005;6:150-166

45. Yoshida K, Mizukami Y, Kitakaze M. Nitric oxide mediates protein kinase c isoform translocation in rat heart during postischemic reperfusion. *Biochim Biophys Acta*. 1999;1453:230-238

46. Matheis G, Sherman MP, Buckberg GD, Haybron DM, Young HH, Ignarro LJ. Role of l-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am J Physiol*. 1992;262:H616-620

47. Pernow J, Wang QD. The role of the l-arginine/nitric oxide pathway in myocardial ischaemic and reperfusion injury. *Acta Physiol Scand*. 1999;167:151-159

48. Zweier JL, Wang P, Kuppusamy P. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J Biol Chem*. 1995;270:304-307

49. Csonka C, Szilvassy Z, Fulop F, Pali T, Blasig IE, Tosaki A, Schulz R, Ferdinandy P. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation*. 1999;100:2260-2266

50. Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *J Biol Chem*. 1996;271:29223-29230

51. Giraldez RR, Panda A, Xia Y, Sanders SP, Zweier JL. Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J Biol Chem*. 1997;272:21420-21426

52. Dumitrescu C, Biondi R, Xia Y, Cardounel AJ, Druhan LJ, Ambrosio G, Zweier JL. Myocardial ischemia results in tetrahydrobiopterin (bh4) oxidation with impaired endothelial function ameliorated by bh4. *Proc Natl Acad Sci U S A*. 2007;104:15081-15086

53. Depre C, Hue L. Cyclic gmp in the perfused rat heart. Effect of ischaemia, anoxia and nitric oxide synthase inhibitor. *FEBS Lett*. 1994;345:241-245

54. Padilla F, Garcia-Dorado D, Agullo L, Barrabes JA, Inserte J, Escalona N, Meyer M, Mirabet M, Pina P, Soler-Soler J. Intravenous administration of the natriuretic peptide urodilatin at low doses during coronary reperfusion limits infarct size in anesthetized pigs. *Cardiovasc Res*. 2001;51:592-600

55. Pernow J, Uriuda Y, Wang QD, Li XS, Nordlander R, Rydeen L. The protective effect of l-arginine on myocardial injury and endothelial function following ischaemia and reperfusion in the pig. *Eur Heart J*. 1994;15:1712-1719

56. Flogel U, Decking UK, Godecke A, Schrader J. Contribution of no to ischemia-reperfusion injury in the saline-perfused heart: A study in endothelial no synthase knockout mice. *J Mol Cell Cardiol*. 1999;31:827-836

57. Weyrich AS, Ma XL, Lefer AM. The role of l-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation*. 1992;86:279-288

58. Sato H, Zhao ZQ, McGee DS, Williams MW, Hammon JW, Jr., Vinten-Johansen J. Supplemental l-arginine during cardioplegic arrest and reperfusion avoids regional postischemic injury. *J Thorac Cardiovasc Surg*. 1995;110:302-314

59. Nakanishi K, Vinten-Johansen J, Lefer DJ, Zhao Z, Fowler WC, 3rd, McGee DS, Johnston WE. Intracoronary l-arginine during reperfusion improves endothelial function and reduces infarct size. *Am J Physiol*. 1992;263:H1650-1658

60. Andelova E, Bartekova M, Pancza D, Styk J, Ravingerova T. The role of no in ischemia/reperfusion injury in isolated rat heart. *Gen Physiol Biophys*. 2005;24:411-426

61. Patel VC, Yellon DM, Singh KJ, Neild GH, Woolfson RG. Inhibition of nitric oxide limits infarct size in the in situ rabbit heart. *Biochem Biophys Res Commun*. 1993;194:234-238

62. Schulz R, Wambolt R. Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovasc Res*. 1995;30:432-439

63. Woolfson RG, Patel VC, Neild GH, Yellon DM. Inhibition of nitric oxide synthesis reduces infarct size by an adenosine-dependent mechanism. *Circulation*. 1995;91:1545-1551

64. Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourd'Heuil D, Huang PL, Lefer DJ. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. *Am J Physiol*. 1999;276:H1567-1573

65. Elrod JW, Greer JJ, Bryan NS, Langston W, Szot JF, Gebregzlabher H, Janssens S, Feelisch M, Lefer DJ. Cardiomyocyte-specific overexpression of no synthase-3 protects against myocardial ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol*. 2006;26:1517-1523

66. du Toit EF, Genade S, Carlini S, Moolman JA, Brunner F, Lochner A. Efficacy of ischaemic preconditioning in the enos overexpressed working mouse heart model. *Eur J Pharmacol*. 2007;556:115-120

67. Sumeray MS, Rees DD, Yellon DM. Infarct size and nitric oxide synthase in murine myocardium. *J Mol Cell Cardiol*. 2000;32:35-42

68. Bell RM, Yellon DM. The contribution of endothelial nitric oxide synthase to early ischaemic preconditioning: The lowering of the preconditioning threshold. An investigation in enos knockout mice. *Cardiovasc Res*. 2001;52:274-280

69. Fleming I. Molecular mechanisms underlying the activation of enos. *Pflugers Arch*. 2010;459:793-806

70. Forstermann U, Sessa WC. Nitric oxide synthases: Regulation and function. *Eur Heart J*. 2012;33:829-837, 837a-837d

71. Fleming I, Busse R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am J Physiol Regul Integr Comp Physiol*. 2003;284:R1-12

72. Harris MB, Ju H, Venema VJ, Liang H, Zou R, Michell BJ, Chen ZP, Kemp BE, Venema RC. Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. *J Biol Chem*. 2001;276:16587-16591

73. Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, Busse R. Phosphorylation of thr(495) regulates ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res*. 2001;88:E68-75

74. Fleming I, Bauersachs J, Fisslthaler B, Busse R. Ca2+-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ Res*. 1998;82:686-695

75. Garcia-Cardena G, Fan R, Stern DF, Liu J, Sessa WC. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J Biol Chem*. 1996;271:27237-27240

76. Fulton D, Church JE, Ruan L, Li C, Sood SG, Kemp BE, Jennings IG, Venema RC. Src kinase activates endothelial nitric-oxide synthase by phosphorylating tyr-83. *J Biol Chem*. 2005;280:35943-35952

77. Fisslthaler B, Dimmeler S, Hermann C, Busse R, Fleming I. Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. *Acta Physiol Scand*. 2000;168:81-88

78. Takenouchi Y, Oo ML, Senga T, Watanabe Y, Machida K, Miyazaki K, Nimura Y, Hamaguchi M. Tyrosine phosphorylation of nos3 in a breast cancer cell line and src-transformed cells. *Oncol Rep*. 2004;11:1059-1062

79. Fulton D, Ruan L, Sood SG, Li C, Zhang Q, Venema RC. Agonist-stimulated endothelial nitric oxide synthase activation and vascular relaxation. Role of enos phosphorylation at tyr83. *Circ Res*. 2008;102:497-504

80. Fisslthaler B, Loot AE, Mohamed A, Busse R, Fleming I. Inhibition of endothelial nitric oxide synthase activity by proline-rich tyrosine kinase 2 in response to fluid shear stress and insulin. *Circ Res*. 2008;102:1520-1528

81. Heusch G. Molecular basis of cardioprotection: Signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res*. 2015;116:674-699

82. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol*. 2003;138:532-543

83. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol*. 2006;40:16-23

84. Kupatt C, Hinkel R, Vachenauer R, Horstkotte J, Raake P, Sandner T, Kreuzpointner R, Müller F, Dimmeler S, Feron O, Boekstegers P. Vegf165 transfection decreases postischemic nf-κb-dependent myocardial reperfusion injury in vivo: Role of enos phosphorylation. *FASEB J*. 2003

85. Sun J, Aponte AM, Kohr MJ, Tong G, Steenbergen C, Murphy E. Essential role of nitric oxide in acute ischemic preconditioning: S-nitros(yl)ation versus sgc/cgmp/pkg signaling? *Free Rad Biol Med*. 2013;54:105-112

86. Garcia-Dorado D, Agulló L, Sartorio CL, Ruiz-Meana M. Myocardial protection against reperfusion injury: The cgmp pathway. *Thromb Haemost*. 2009;101:635-642

87. Burley DS, Ferdinandy P, Baxter GF. Cyclic gmp and protein kinase-g in myocardial ischaemia-reperfusion: Opportunities and obstacles for survival signaling. *Br J Pharmacol*. 2007;152:855-869

88. Inserte J, Ruiz-Meana M, Rodríguez-Sinovas A, Barba I, Garcia-Dorado D. Contribution of delayed intracellular ph recovery to ischemic postconditioning protection. *Antioxid Redox Signal*. 2010;14:923-939

89. Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, James AM, Cocheme HM, Reinhold J, Lilley KS, Partridge L, Fearnley IM, Robinson AJ, Hartley RC, Smith RAJ, Krieg T, Brookes PS, Murphy MP. Cardioprotection by s-nitrosation of a cysteine switch on mitochondrial complex i. *Nat Med*. 2013;19:753-759

90. Cai M, Li Y, Xu Y, Swartz HM, Chen CL, Chen YR, He G. Endothelial nos activity and myocardial oxygen metabolism define the salvageable ischemic time window for ischemic postconditioning. *Am J Physiol Heart Circ Physiol*. 2011;300:H1069-1077

91. Inserte J, Hernando V, Vilardosa U, Abad E, Poncelas-Nozal M, Garcia-Dorado D. Activation of cgmp/protein kinase g pathway in postconditioned myocardium depends on

reduced oxidative stress and preserved endothelial nitric oxide synthase coupling. *J Am Heart Assoc*. 2013;2:e005975

92. Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR, Kemp BE. Amp-activated protein kinase phosphorylation of endothelial no synthase. *FEBS Lett*. 1999;443:285-289

93. Avraham H, Park SY, Schinkmann K, Avraham S. Raftk/pyk2-mediated cellular signalling. *Cell Signal*. 2000;12:123-133

94. Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci U S A*. 1987;84:1404-1407

95. Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, McCay PB. Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc Natl Acad Sci U S A*. 1989;86:4695-4699

96. Zweier JL, Kuppusamy P, Lutty GA. Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissues. *Proc Natl Acad Sci U S A*. 1988;85:4046-4050

97. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res*. 2006;70:181-190

98. Pong T, Scherrer-Crosbie M, Atochin DN, Bloch KD, Huang PL. Phosphomimetic modulation of enos improves myocardial reperfusion and mimics cardiac postconditioning in mice. *PLoS One*. 2014;9:e85946

99. Kupatt C, Hinkel R, Vachenauer R, Horstkotte J, Raake P, Sandner T, Kreuzpointner R, Muller F, Dimmeler S, Feron O, Boekstegers P. Vegf165 transfection decreases postischemic nf-kappa b-dependent myocardial reperfusion injury in vivo: Role of enos phosphorylation. *FASEB J*. 2003;17:705-707

100. Zhao ZQ, Nakamura M, Wang NP, Velez DA, Hewan-Lowe KO, Guyton RA, Vinten-Johansen J. Dynamic progression of contractile and endothelial dysfunction and infarct extension in the late phase of reperfusion. *J Surg Res*. 2000;94:133-144

101. Heusch G. Molecular basis of cardioprotection: Signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res*. 2015;116:674-699

102. Maroko PR, Libby P, Ginks WR, Bloor CM, Shell WE, Sobel BE, Ross J, Jr. Coronary artery reperfusion. I. Early effects on local myocardial function and the extent of myocardial necrosis. *J Clin Invest*. 1972;51:2710-2716

103. Ginks WR, Sybers HD, Maroko PR, Covell JW, Sobel BE, Ross J, Jr. Coronary artery reperfusion. Ii. Reduction of myocardial infarct size at 1 week after the coronary occlusion. *J Clin Invest*. 1972;51:2717-2723

104. Reimer KA, Lowe JE, Rasmussen MM, Jennings RB. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. *Circulation*. 1977;56:786-794

105. Reimer KA, Jennings RB. The "wavefront phenomenon" of myocardial ischemic cell death. Ii. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. *Lab Invest*. 1979;40:633-644

106. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124-1136

107. Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M, Inserte J. Protection against myocardial ischemia-reperfusion injury in clinical practice. *Rev Esp Cardiol (Engl Ed)*. 2014;67:394-404

108. Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res*. 1990;66:913-931

109. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by a1 adenosine receptors in rabbit heart. *Circulation*. 1991;84:350-356

110. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation*. 1993;88:1264-1272

111. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res*. 1993;72:1293-1299

112. Bolli R. The late phase of preconditioning. *Circ Res*. 2000;87:972-983

113. Heusch G. Cardioprotection: Chances and challenges of its translation to the clinic. *Lancet*. 2013;381:166-175

114. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: Comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol*. 2003;285:H579-588

115. Przyklenk K. Lethal myocardial "reperfusion injury": The opinions of good men. *J Thromb Thrombolysis*. 1997;4:5-6

116. Heusch G. Postconditioning: Old wine in a new bottle? *J Am Coll Cardiol*. 2004;44:1111-1112

117. Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacol Rev*. 2014;66:1142-1174

118. Ashraf M, Suleiman J, Ahmad M. Ca2+ preconditioning elicits a unique protection against the ca2+ paradox injury in rat heart. Role of adenosine. Fixed. *Circ Res*. 1994;74:360-367

119. Miyawaki H, Zhou X, Ashraf M. Calcium preconditioning elicits strong protection against ischemic injury via protein kinase c signaling pathway. *Circ Res*. 1996;79:137-146

120. Tullio F, Angotti C, Perrelli MG, Penna C, Pagliaro P. Redox balance and cardioprotection. *Basic Res Cardiol*. 2013;108:392

121. Rassaf T, Poll LW, Brouzos P, Lauer T, Totzeck M, Kleinbongard P, Gharini P, Andersen K, Schulz R, Heusch G, Modder U, Kelm M. Positive effects of nitric oxide on left ventricular function in humans. *Eur Heart J*. 2006;27:1699-1705

122. Heusch G, Post H, Michel MC, Kelm M, Schulz R. Endogenous nitric oxide and myocardial adaptation to ischemia. *Circ Res*. 2000;87:146-152

123. Heinzel FR, Gres P, Boengler K, Duschin A, Konietzka I, Rassaf T, Snedovskaya J, Meyer S, Skyschally A, Kelm M, Heusch G, Schulz R. Inducible nitric oxide synthase expression and cardiomyocyte dysfunction during sustained moderate ischemia in pigs. *Circ Res*. 2008;103:1120-1127

124. Schulz R, Post H, Vahlhaus C, Heusch G. Ischemic preconditioning in pigs: A graded phenomenon: Its relation to adenosine and bradykinin. *Circulation*. 1998;98:1022-1029

125. Schulz R, Rose J, Post H, Heusch G. Involvement of endogenous adenosine in ischaemic preconditioning in swine. *Pflugers Arch*. 1995;430:273-282

126. Downey JM, Davis AM, Cohen MV. Signaling pathways in ischemic preconditioning. *Heart Fail Rev*. 2007;12:181-188

127. Heusch G, Boengler K, Schulz R. Cardioprotection: Nitric oxide, protein kinases, and mitochondria. *Circulation*. 2008;118:1915-1919

128. Grube K, Rudebusch J, Xu Z, Bockenholt T, Methner C, Muller T, Cuello F, Zimmermann K, Yang X, Felix SB, Cohen MV, Downey JM, Krieg T. Evidence for an intracellular localization of the adenosine a2b receptor in rat cardiomyocytes. *Basic Res Cardiol*. 2011;106:385-396

129. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine a3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res*. 1994;28:1057-1061

130. Methner C, Schmidt K, Cohen MV, Downey JM, Krieg T. Both a2a and a2b adenosine receptors at reperfusion are necessary to reduce infarct size in mouse hearts. *Am J Physiol Heart Circ Physiol*. 2010;299:H1262-1264

131. Bibli SI, Iliodromitis EK, Lambertucci C, Zoga A, Lougiakis N, Dagres N, Volpini R, Dal Ben D, Kremastinos DT, Tsantili Kakoulidou A, Cristalli G, Andreadou I. Pharmacological postconditioning of the rabbit heart with non-selective, a1 , a2a and a3 adenosine receptor agonists. *J Pharm Pharmacol*. 2014;66:1140-1149

132. Kleinbongard P, Heusch G. Extracellular signalling molecules in the ischaemic/reperfused heart - druggable and translatable for cardioprotection? *Br J Pharmacol*. 2015;172:2010-2025

133. Kokkonen JO, Lindstedt KA, Kuoppala A, Kovanen PT. Kinin-degrading pathways in the human heart. *Trends Cardiovasc Med*. 2000;10:42-45

134. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ Res*. 1995;77:611-621

135. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial k(atp) channels. *Circ Res*. 2001;89:273-278

136. Salie R, Moolman JA, Lochner A. The mechanism of beta-adrenergic preconditioning: Roles for adenosine and ros during triggering and mediation. *Basic Res Cardiol*. 2012;107:281

137. Kudej RK, Shen YT, Peppas AP, Huang CH, Chen W, Yan L, Vatner DE, Vatner SF. Obligatory role of cardiac nerves and alpha1-adrenergic receptors for the second window of ischemic preconditioning in conscious pigs. *Circ Res*. 2006;99:1270-1276

138. Schulz R, Gres P, Heusch G. Role of endogenous opioids in ischemic preconditioning but not in short-term hibernation in pigs. *Am J Physiol Heart Circ Physiol*. 2001;280:H2175-2181

139. Zatta AJ, Kin H, Yoshishige D, Jiang R, Wang N, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Caffrey JL, Vinten-Johansen J. Evidence that cardioprotection by postconditioning involves preservation of myocardial opioid content and selective opioid receptor activation. *Am J Physiol Heart Circ Physiol*. 2008;294:H1444-1451

140. Penna C, Mancardi D, Tullio F, Pagliaro P. Postconditioning and intermittent bradykinin induced cardioprotection require cyclooxygenase activation and prostacyclin release during reperfusion. *Basic Res Cardiol*. 2008;103:368-377

141. Gross GJ, Gauthier KM, Moore J, Campbell WB, Falck JR, Nithipatikom K. Evidence for role of epoxyeicosatrienoic acids in mediating ischemic preconditioning and postconditioning in dog. *Am J Physiol Heart Circ Physiol*. 2009;297:H47-52

142. Jin ZQ, Goetzl EJ, Karliner JS. Sphingosine kinase activation mediates ischemic preconditioning in murine heart. *Circulation*. 2004;110:1980-1989

143. Jin ZQ, Karliner JS, Vessey DA. Ischaemic postconditioning protects isolated mouse hearts against ischaemia/reperfusion injury via sphingosine kinase isoform-1 activation. *Cardiovasc Res*. 2008;79:134-140

144. Gomez L, Paillard M, Price M, Chen Q, Teixeira G, Spiegel S, Lesnefsky EJ. A novel role for mitochondrial sphingosine-1-phosphate produced by sphingosine kinase-2 in ptp-mediated cell survival during cardioprotection. *Basic Res Cardiol*. 2011;106:1341-1353

145. Lecour S, Suleman N, Deuchar GA, Somers S, Lacerda L, Huisamen B, Opie LH. Pharmacological preconditioning with tumor necrosis factor-alpha activates signal transducer and activator of transcription-3 at reperfusion without involving classic prosurvival kinases (akt and extracellular signal-regulated kinase). *Circulation*. 2005;112:3911-3918

146. Smith RM, Suleman N, McCarthy J, Sack MN. Classic ischemic but not pharmacologic preconditioning is abrogated following genetic ablation of the tnfalpha gene. *Cardiovasc Res*. 2002;55:553-560

147. Lacerda L, Somers S, Opie LH, Lecour S. Ischaemic postconditioning protects against reperfusion injury via the safe pathway. *Cardiovasc Res*. 2009;84:201-208

148. Liu Y, Cohen MV, Downey JM. Chelerythrine, a highly selective protein kinase c inhibitor, blocks the anti-infarct effect of ischemic preconditioning in rabbit hearts. *Cardiovasc Drugs Ther*. 1994;8:881-882

149. Vahlhaus C, Schulz R, Post H, Rose J, Heusch G. Prevention of ischemic preconditioning only by combined inhibition of protein kinase c and protein tyrosine kinase in pigs. *J Mol Cell Cardiol*. 1998;30:197-209

150. Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase c by nitric oxide in the heart of conscious rabbits: A signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ Res*. 1999;84:587-604

151. Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Messing RO, Bolli R. Role of the protein kinase c-epsilon-raf-1-mek-1/2-p44/42 mapk signaling cascade in the activation of signal transducers and activators of transcription 1 and 3 and induction of cyclooxygenase-2 after ischemic preconditioning. *Circulation*. 2005;112:1971-1978

152. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase c isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase c activity. *Circ Res*. 1997;81:404-414

153. Ping P, Song C, Zhang J, Guo Y, Cao X, Li RC, Wu W, Vondriska TM, Pass JM, Tang XL, Pierce WM, Bolli R. Formation of protein kinase c(epsilon)-lck signaling modules confers cardioprotection. *J Clin Invest*. 2002;109:499-507

154. Budas GR, Churchill EN, Disatnik MH, Sun L, Mochly-Rosen D. Mitochondrial import of pkcepsilon is mediated by hsp90: A role in cardioprotection from ischaemia and reperfusion injury. *Cardiovasc Res*. 2010;88:83-92

155. Ohnuma Y, Miura T, Miki T, Tanno M, Kuno A, Tsuchida A, Shimamoto K. Opening of mitochondrial k(atp) channel occurs downstream of pkc-epsilon activation in the mechanism of preconditioning. *Am J Physiol Heart Circ Physiol*. 2002;283:H440-447

156. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial atp-sensitive k+ channel and protein kinase c activation. *Basic Res Cardiol*. 2006;101:180-189

157. Kuno A, Critz SD, Cui L, Solodushko V, Yang XM, Krahn T, Albrecht B, Philipp S, Cohen MV, Downey JM. Protein kinase c protects preconditioned rabbit hearts by increasing sensitivity of adenosine a2b-dependent signaling during early reperfusion. *J Mol Cell Cardiol*. 2007;43:262-271

158. Cohen A. Should we tolerate tolerability as an objective in early drug development? *Br J Clin Pharmacol*. 2007;64:249-252

159. Costa AD, Pierre SV, Cohen MV, Downey JM, Garlid KD. Cgmp signalling in pre- and post-conditioning: The role of mitochondria. *Cardiovasc Res*. 2008;77:344-352

160. Yang C, Talukder MA, Varadharaj S, Velayutham M, Zweier JL. Early ischaemic preconditioning requires akt- and pka-mediated activation of enos via serine1176 phosphorylation. *Cardiovasc Res*. 2013;97:33-43

161. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase akt. *Nature*. 1999;399:597-601

162. Schulz R, Ferdinandy P. Does nitric oxide signaling differ in pre- and post-conditioning? Importance of s-nitrosylation vs. Protein kinase g activation. *Free Radic Biol Med*. 2013;54:113-115

163. Sun J, Aponte AM, Kohr MJ, Tong G, Steenbergen C, Murphy E. Essential role of nitric oxide in acute ischemic preconditioning: S-nitros(yl)ation versus sgc/cgmp/pkg signaling? *Free Radic Biol Med*. 2013;54:105-112

164. Cohen MV, Yang XM, Liu Y, Solenkova NV, Downey JM. Cardioprotective pkg-independent no signaling at reperfusion. *Am J Physiol Heart Circ Physiol*. 2010;299:H2028-2036

165. Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P. Intermittent activation of bradykinin b2 receptors and mitochondrial katp channels trigger cardiac postconditioning through redox signaling. *Cardiovasc Res*. 2007;75:168-177

166. Garcia-Dorado D, Agullo L, Sartorio CL, Ruiz-Meana M. Myocardial protection against reperfusion injury: The cgmp pathway. *Thromb Haemost*. 2009;101:635-642

167. Inserte J, Ruiz-Meana M, Rodriguez-Sinovas A, Barba I, Garcia-Dorado D. Contribution of delayed intracellular ph recovery to ischemic postconditioning protection. *Antioxid Redox Signal*. 2011;14:923-939

168. Inserte J, Hernando V, Ruiz-Meana M, Poncelas-Nozal M, Fernandez C, Agullo L, Sartorio C, Vilardosa U, Garcia-Dorado D. Delayed phospholamban phosphorylation in post-conditioned heart favours ca2+ normalization and contributes to protection. *Cardiovasc Res*. 2014;103:542-553

169. Karczewski P, Kuschel M, Baltas LG, Bartel S, Krause EG. Site-specific phosphorylation of a phospholamban peptide by cyclic nucleotide- and ca2+/calmodulin-dependent protein kinases of cardiac sarcoplasmic reticulum. *Basic Res Cardiol*. 1997;92 Suppl 1:37-43

170. Sabine B, Willenbrock R, Haase H, Karczewski P, Wallukat G, Dietz R, Krause EG. Cyclic gmp-mediated phospholamban phosphorylation in intact cardiomyocytes. *Biochem Biophys Res Commun*. 1995;214:75-80

171. Abdallah Y, Gkatzoflia A, Pieper H, Zoga E, Walther S, Kasseckert S, Schafer M, Schluter KD, Piper HM, Schafer C. Mechanism of cgmp-mediated protection in a cellular model of myocardial reperfusion injury. *Cardiovasc Res*. 2005;66:123-131

172. Gorbe A, Giricz Z, Szunyog A, Csont T, Burley DS, Baxter GF, Ferdinandy P. Role of cgmp-pkg signaling in the protection of neonatal rat cardiac myocytes subjected to simulated ischemia/reoxygenation. *Basic Res Cardiol*. 2010;105:643-650

173. Vittone L, Mundina-Weilenmann C, Said M, Ferrero P, Mattiazzi A. Time course and mechanisms of phosphorylation of phospholamban residues in ischemia-reperfused rat hearts. Dissociation of phospholamban phosphorylation pathways. *J Mol Cell Cardiol*. 2002;34:39-50

174. Kranias EG, Hajjar RJ. Modulation of cardiac contractility by the phospholamban/serca2a regulatome. *Circ Res*. 2012;110:1646-1660

175. Tong H, Chen W, Steenbergen C, Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase c. *Circ Res*. 2000;87:309-315

176. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase--dependent pathway is cardioprotective. *Circ Res*. 2002;90:377-379

177. Krieg T, Qin Q, McIntosh EC, Cohen MV, Downey JM. Ach and adenosine activate pi3-kinase in rabbit hearts through transactivation of receptor tyrosine kinases. *Am J Physiol Heart Circ Physiol*. 2002;283:H2322-2330

178. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. *Am J Physiol Heart Circ Physiol*. 2005;288:H971-976

179. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest*. 2004;113:1535-1549

180. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: A form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-akt pathway. *Circ Res*. 2004;95:230-232

181. Zhu M, Feng J, Lucchinetti E, Fischer G, Xu L, Pedrazzini T, Schaub MC, Zaugg M. Ischemic postconditioning protects remodeled myocardium via the pi3k-pkb/akt reperfusion injury salvage kinase pathway. *Cardiovasc Res*. 2006;72:152-162

182. Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, Yellon DM. Postconditioning protects human atrial muscle through the activation of the risk pathway. *Basic Res Cardiol*. 2007;102:453-459

183. Juhaszova M, Zorov DB, Yaniv Y, Nuss HB, Wang S, Sollott SJ. Role of glycogen synthase kinase-3beta in cardioprotection. *Circ Res*. 2009;104:1240-1252

184. Ong SB, Hall AR, Dongworth RK, Kalkhoran S, Pyakurel A, Scorrano L, Hausenloy DJ. Akt protects the heart against ischaemia-reperfusion injury by modulating mitochondrial morphology. *Thromb Haemost*. 2015;113:513-521

185. Nishino Y, Webb IG, Davidson SM, Ahmed AI, Clark JE, Jacquet S, Shah AM, Miura T, Yellon DM, Avkiran M, Marber MS. Glycogen synthase kinase-3 inactivation is not required for ischemic preconditioning or postconditioning in the mouse. *Circ Res*. 2008;103:307-314

186. Gomez L, Paillard M, Thibault H, Derumeaux G, Ovize M. Inhibition of gsk3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. *Circulation*. 2008;117:2761-2768

187. Skyschally A, van Caster P, Boengler K, Gres P, Musiolik J, Schilawa D, Schulz R, Heusch G. Ischemic postconditioning in pigs: No causal role for risk activation. *Circ Res*. 2009;104:15-18

188. Smith RM, Suleman N, Lacerda L, Opie LH, Akira S, Chien KR, Sack MN. Genetic depletion of cardiac myocyte stat-3 abolishes classical preconditioning. *Cardiovasc Res*. 2004;63:611-616

189. Suleman N, Somers S, Smith R, Opie LH, Lecour SC. Dual activation of stat-3 and akt is required during the trigger phase of ischaemic preconditioning. *Cardiovasc Res*. 2008;79:127-133

190. Huffman LC, Koch SE, Butler KL. Coronary effluent from a preconditioned heart activates the jak-stat pathway and induces cardioprotection in a donor heart. *Am J Physiol Heart Circ Physiol*. 2008;294:H257-262

191. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R. Cardioprotection by ischemic postconditioning is lost in aged and stat3-deficient mice. *Circ Res*. 2008;102:131-135

192. Heusch G, Musiolik J, Gedik N, Skyschally A. Mitochondrial stat3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. *Circ Res*. 2011;109:1302-1308

193. Lecour S. Activation of the protective survivor activating factor enhancement (safe) pathway against reperfusion injury: Does it go beyond the risk pathway? *J Mol Cell Cardiol*. 2009;47:32-40

194. Bolli R, Stein AB, Guo Y, Wang OL, Rokosh G, Dawn B, Molkentin JD, Sanganalmath SK, Zhu Y, Xuan YT. A murine model of inducible, cardiac-specific deletion of stat3: Its use to determine the role of stat3 in the upregulation of cardioprotective proteins by ischemic preconditioning. *J Mol Cell Cardiol*. 2011;50:589-597

195. Wegrzyn J, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC. Function of mitochondrial stat3 in cellular respiration. *Science*. 2009;323:793-797

196. Weiss JB, Eisenhardt SU, Stark GB, Bode C, Moser M, Grundmann S. Micrornas in ischemia-reperfusion injury. *Am J Cardiovasc Dis*. 2012;2:237-247

197. Yin C, Salloum FN, Kukreja RC. A novel role of microrna in late preconditioning: Upregulation of endothelial nitric oxide synthase and heat shock protein 70. *Circ Res*. 2009;104:572-575

198. Cheng Y, Zhu P, Yang J, Liu X, Dong S, Wang X, Chun B, Zhuang J, Zhang C. Ischaemic preconditioning-regulated mir-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target pdcd4. *Cardiovasc Res*. 2010;87:431-439

199. Wang X, Zhu H, Zhang X, Liu Y, Chen J, Medvedovic M, Li H, Weiss MJ, Ren X, Fan GC. Loss of the mir-144/451 cluster impairs ischaemic preconditioning-mediated cardioprotection by targeting rac-1. *Cardiovasc Res*. 2012;94:379-390

200. Varga ZV, Zvara A, Farago N, Kocsis GF, Pipicz M, Gaspar R, Bencsik P, Gorbe A, Csonka C, Puskas LG, Thum T, Csont T, Ferdinandy P. Micrornas associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: Protectomirs. *Am J Physiol Heart Circ Physiol*. 2014;307:H216-227

201. Baars T, Skyschally A, Klein-Hitpass L, Cario E, Erbel R, Heusch G, Kleinbongard P. Microrna expression and its potential role in cardioprotection by ischemic postconditioning in pigs. *Pflugers Arch*. 2014;466:1953-1961

202. Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, Wei C, Hu P, Kharbanda RK, Redington AN. Microrna-144 is a circulating effector of remote ischemic preconditioning. *Basic Res Cardiol*. 2014;109:423

203. Slagsvold KH, Rognmo O, Hoydal M, Wisloff U, Wahba A. Remote ischemic preconditioning preserves mitochondrial function and influences myocardial microrna expression in atrial myocardium during coronary bypass surgery. *Circ Res*. 2014;114:851-859

204. Bernardi P, Di Lisa F. The mitochondrial permeability transition pore: Molecular nature and role as a target in cardioprotection. *J Mol Cell Cardiol*. 2015;78:100-106

205. Heusch G, Boengler K, Schulz R. Inhibition of mitochondrial permeability transition pore opening: The holy grail of cardioprotection. *Basic Res Cardiol*. 2010;105:151-154

206. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC. The adp/atp translocator is not essential for the mitochondrial permeability transition pore. *Nature*. 2004;427:461-465

207. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabo I, Lippe G, Bernardi P. Dimers of mitochondrial atp synthase form the permeability transition pore. *Proc Natl Acad Sci U S A*. 2013;110:5887-5892

208. Hausenloy DJ, Duchen MR, Yellon DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. *Cardiovasc Res*. 2003;60:617-625

209. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ros) and ros-induced ros release. *Physiol Rev*. 2014;94:909-950

210. Hausenloy D, Wynne A, Duchen M, Yellon D. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation*. 2004;109:1714-1717

211. Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R. Inhibition of permeability transition pore opening by mitochondrial stat3 and its role in myocardial ischemia/reperfusion. *Basic Res Cardiol*. 2010;105:771-785

212. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, Andre-Fouet X, Revel D, Kirkorian G, Monassier JP, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med*. 2008;359:473-481

213. Grover GJ, Dzwonczyk S, Sleph PG, Sargent CA. The atp-sensitive potassium channel blocker glibenclamide (glyburide) does not abolish preconditioning in isolated ischemic rat hearts. *J Pharmacol Exp Ther*. 1993;265:559-564

214. Schulz R, Rose J, Heusch G. Involvement of activation of atp-dependent potassium channels in ischemic preconditioning in swine. *Am J Physiol*. 1994;267:H1341-1352

215. Suzuki M, Saito T, Sato T, Tamagawa M, Miki T, Seino S, Nakaya H. Cardioprotective effect of diazoxide is mediated by activation of sarcolemmal but not mitochondrial atp-sensitive potassium channels in mice. *Circulation*. 2003;107:682-685

216. Ardehali H, O'Rourke B. Mitochondrial k(atp) channels in cell survival and death. *J Mol Cell Cardiol*. 2005;39:7-16

217. Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. Activation of mitochondrial atp-dependent potassium channels by nitric oxide. *Circulation*. 2000;101:439-445

218. Costa AD, Jakob R, Costa CL, Andrukhiv K, West IC, Garlid KD. The mechanism by which the mitochondrial atp-sensitive k+ channel opening and h2o2 inhibit the mitochondrial permeability transition. *J Biol Chem*. 2006;281:20801-20808

219. Costa AD, Garlid KD, West IC, Lincoln TM, Downey JM, Cohen MV, Critz SD. Protein kinase g transmits the cardioprotective signal from cytosol to mitochondria. *Circ Res*. 2005;97:329-336

220. Jaburek M, Costa AD, Burton JR, Costa CL, Garlid KD. Mitochondrial pkc epsilon and mitochondrial atp-sensitive k+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. *Circ Res*. 2006;99:878-883

221. Kohr MJ, Sun J, Aponte A, Wang G, Gucek M, Murphy E, Steenbergen C. Simultaneous measurement of protein oxidation and s-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture. *Circ Res*. 2011;108:418-426

222. Methner C, Lukowski R, Grube K, Loga F, Smith RA, Murphy MP, Hofmann F, Krieg T. Protection through postconditioning or a mitochondria-targeted s-nitrosothiol is unaffected by cardiomyocyte-selective ablation of protein kinase g. *Basic Res Cardiol*. 2013;108:337

223. Cohen MV, Yang XM, Downey JM. The ph hypothesis of postconditioning: Staccato reperfusion reintroduces oxygen and perpetuates myocardial acidosis. *Circulation*. 2007;115:1895-1903

224. Hausenloy DJ, Wynne AM, Yellon DM. Ischemic preconditioning targets the reperfusion phase. *Basic Res Cardiol*. 2007;102:445-452

225. Xiao XH, Allen DG. Activity of the na(+)/h(+) exchanger is critical to reperfusion damage and preconditioning in the isolated rat heart. *Cardiovasc Res*. 2000;48:244-253

226. Cohen MV, Yang XM, Downey JM. Acidosis, oxygen, and interference with mitochondrial permeability transition pore formation in the early minutes of reperfusion are critical to postconditioning's success. *Basic Res Cardiol*. 2008;103:464-471

227. Inserte J, Barba I, Hernando V, Garcia-Dorado D. Delayed recovery of intracellular acidosis during reperfusion prevents calpain activation and determines protection in postconditioned myocardium. *Cardiovasc Res*. 2009;81:116-122

228. Inserte J, Barba I, Poncelas-Nozal M, Hernando V, Agullo L, Ruiz-Meana M, Garcia-Dorado D. Cgmp/pkg pathway mediates myocardial postconditioning protection in rat hearts by delaying normalization of intracellular acidosis during reperfusion. *J Mol Cell Cardiol*. 2011;50:903-909

229. Thuny F, Lairez O, Roubille F, Mewton N, Rioufol G, Sportouch C, Sanchez I, Bergerot C, Thibault H, Cung TT, Finet G, Argaud L, Revel D, Derumeaux G, Bonnefoy-Cudraz E, Elbaz M, Piot C, Ovize M, Croisille P. Post-conditioning reduces infarct size and edema in patients with st-segment elevation myocardial infarction. *J Am Coll Cardiol*. 2012;59:2175-2181

230. Hahn JY, Song YB, Kim EK, Yu CW, Bae JW, Chung WY, Choi SH, Choi JH, Bae JH, An KJ, Park JS, Oh JH, Kim SW, Hwang JY, Ryu JK, Park HS, Lim DS, Gwon HC. Ischemic postconditioning during primary percutaneous coronary intervention: The effects of postconditioning on myocardial reperfusion in patients with st-segment elevation myocardial infarction (post) randomized trial. *Circulation*. 2013;128:1889-1896

231. Thielmann M, Kottenberg E, Kleinbongard P, Wendt D, Gedik N, Pasa S, Price V, Tsagakis K, Neuhauser M, Peters J, Jakob H, Heusch G. Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: A single-centre randomised, double-blind, controlled trial. *Lancet*. 2013;382:597-604

232. Monassier L, Ayme-Dietrich E, Aubertin-Kirch G, Pathak A. Targeting myocardial reperfusion injuries with cyclosporine in the circus trial - pharmacological reasons for failure. *Fundam Clin Pharmacol*. 2015

233. Hausenloy DJ, Erik Botker H, Condorelli G, Ferdinandy P, Garcia-Dorado D, Heusch G, Lecour S, van Laake LW, Madonna R, Ruiz-Meana M, Schulz R, Sluijter JP, Yellon DM, Ovize M. Translating cardioprotection for patient benefit: Position paper from the working group of cellular biology of the heart of the european society of cardiology. *Cardiovasc Res*. 2013;98:7-27

234. Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, Heusch G, Vinten-Johansen J, Yellon DM, Schulz R. Postconditioning and protection from reperfusion injury: Where do we stand? Position paper from the working group of cellular biology of the heart of the european society of cardiology. *Cardiovasc Res*. 2010;87:406-423

235. Bell R, Beeuwkes R, Botker HE, Davidson S, Downey J, Garcia-Dorado D, Hausenloy DJ, Heusch G, Ibanez B, Kitakaze M, Lecour S, Mentzer R, Miura T, Opie L, Ovize M, Ruiz-Meana M, Schulz R, Shannon R, Walker M, Vinten-Johansen J, Yellon D. Trials, tribulations and speculation! Report from the 7th biennial hatter cardiovascular institute workshop. *Basic Res Cardiol*. 2012;107:300

236. Ferdinandy P, Schulz R, Baxter GF. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev*. 2007;59:418-458

237. Shinbo T, Kokubo K, Sato Y, Hagiri S, Hataishi R, Hirose M, Kobayashi H. Breathing nitric oxide plus hydrogen gas reduces ischemia-reperfusion injury and nitrotyrosine production in murine heart. *Am J Physiol Heart Circ Physiol*. 2013;305:H542-550

238. Nunez C, Victor VM, Tur R, Alvarez-Barrientos A, Moncada S, Esplugues JV, D'Ocon P. Discrepancies between nitroglycerin and no-releasing drugs on mitochondrial oxygen consumption, vasoactivity, and the release of no. *Circ Res*. 2005;97:1063-1069

239. Kojima S, Matsui K, Sakamoto T, Ishihara M, Kimura K, Miyazaki S, Yamagishi M, Tei C, Hiraoka H, Sonoda M, Tsuchihashi K, Shimoyama N, Honda T, Ogata Y, Ogawa H. Long-term nitrate therapy after acute myocardial infarction does not improve or aggravate prognosis. *Circ J*. 2007;71:301-307

240. Munzel T, Gori T. Nitrate therapy and nitrate tolerance in patients with coronary artery disease. *Curr Opin Pharmacol*. 2013;13:251-259

241. Csont T, Ferdinandy P. Cardioprotective effects of glyceryl trinitrate: Beyond vascular nitrate tolerance. *Pharmacol Ther*. 2005;105:57-68

242. Gori T, Dragoni S, Di Stolfo G, Sicuro S, Liuni A, Luca MC, Thomas G, Oelze M, Daiber A, Parker JD. Tolerance to nitroglycerin-induced preconditioning of the endothelium: A human in vivo study. *Am J Physiol Heart Circ Physiol*. 2010;298:H340-345

243. Lisi M, Oelze M, Dragoni S, Liuni A, Steven S, Luca MC, Stalleicken D, Munzel T, Laghi-Pasini F, Daiber A, Parker JD, Gori T. Chronic protection against ischemia and reperfusion-induced endothelial dysfunction during therapy with different organic nitrates. *Clin Res Cardiol*. 2012;101:453-459

244. Iliodromitis EK, Gaitanaki C, Lazou A, Aggeli IK, Gizas V, Bofilis E, Zoga A, Beis I, Kremastinos DT. Differential activation of mitogen-activated protein kinases in ischemic and nitroglycerin-induced preconditioning. *Basic Res Cardiol*. 2006;101:327-335

245. Liu X, Huang Y, Pokreisz P, Vermeersch P, Marsboom G, Swinnen M, Verbeken E, Santos J, Pellens M, Gillijns H, Van de Werf F, Bloch KD, Janssens S. Nitric oxide inhalation improves microvascular flow and decreases infarction size after myocardial ischemia and reperfusion. *J Am Coll Cardiol*. 2007;50:808-817

246. Salloum FN, Takenoshita Y, Ockaili RA, Daoud VP, Chou E, Yoshida K, Kukreja RC. Sildenafil and vardenafil but not nitroglycerin limit myocardial infarction through opening of mitochondrial k(atp) channels when administered at reperfusion following ischemia in rabbits. *J Mol Cell Cardiol*. 2007;42:453-458

247. Mizumura T, Nithipatikom K, Gross GJ. Effects of nicorandil and glyceryl trinitrate on infarct size, adenosine release, and neutrophil infiltration in the dog. *Cardiovasc Res*. 1995;29:482-489

248. Andreadou I, Farmakis D, Prokovas E, Sigala F, Zoga A, Spyridaki K, Papalois A, Papapetropoulos A, Anastasiou-Nana M, Kremastinos DT, Iliodromitis EK. Short-term statin administration in hypercholesterolaemic rabbits resistant to postconditioning: Effects on infarct size, endothelial nitric oxide synthase, and nitro-oxidative stress. *Cardiovasc Res*. 2012;94:501-509

249. Zhang Z, Huang H, Liu P, Tang C, Wang J. Hydrogen sulfide contributes to cardioprotection during ischemia-reperfusion injury by opening k atp channels. *Can J Physiol Pharmacol*. 2007;85:1248-1253

250. Ji Y, Pang QF, Xu G, Wang L, Wang JK, Zeng YM. Exogenous hydrogen sulfide postconditioning protects isolated rat hearts against ischemia-reperfusion injury. *Eur J Pharmacol*. 2008;587:1-7

251. Johansen D, Ytrehus K, Baxter GF. Exogenous hydrogen sulfide (h2s) protects against regional myocardial ischemia-reperfusion injury--evidence for a role of k atp channels. *Basic Res Cardiol*. 2006;101:53-60

252. Abdelnoor M, Sandven I, Limalanathan S, Eritsland J. Postconditioning in st-elevation myocardial infarction: A systematic review, critical appraisal, and meta-analysis of randomized clinical trials. *Vasc Health Risk Manag*. 2014;10:477-491

253. Brevoord D, Kranke P, Kuijpers M, Weber N, Hollmann M, Preckel B. Remote ischemic conditioning to protect against ischemia-reperfusion injury: A systematic review and meta-analysis. *PLoS One*. 2012;7:e42179

254. Tang XL, Takano H, Xuan YT, Sato H, Kodani E, Dawn B, Zhu Y, Shirk G, Wu WJ, Bolli R. Hypercholesterolemia abrogates late preconditioning via a tetrahydrobiopterin-dependent mechanism in conscious rabbits. *Circulation*. 2005;112:2149-2156

255. Iliodromitis EK, Zoga A, Vrettou A, Andreadou I, Paraskevaidis IA, Kaklamanis L, Kremastinos DT. The effectiveness of postconditioning and preconditioning on infarct size in hypercholesterolemic and normal anesthetized rabbits. *Atherosclerosis*. 2006;188:356-362

256. Kupai K, Csonka C, Fekete V, Odendaal L, van Rooyen J, Marais de W, Csont T, Ferdinandy P. Cholesterol diet-induced hyperlipidemia impairs the cardioprotective effect of postconditioning: Role of peroxynitrite. *Am J Physiol Heart Circ Physiol*. 2009;297:H1729-1735

257. Donato M, D'Annunzio V, Berg G, Gonzalez G, Schreier L, Morales C, Wikinski RL, Gelpi RJ. Ischemic postconditioning reduces infarct size by activation of a1 receptors and k+(atp) channels in both normal and hypercholesterolemic rabbits. *J Cardiovasc Pharmacol*. 2007;49:287-292

258. Giricz Z, Lalu MM, Csonka C, Bencsik P, Schulz R, Ferdinandy P. Hyperlipidemia attenuates the infarct size-limiting effect of ischemic preconditioning: Role of matrix metalloproteinase-2 inhibition. *J Pharmacol Exp Ther*. 2006;316:154-161

259. Kocsis GF, Csont T, Varga-Orvos Z, Puskas LG, Murlasits Z, Ferdinandy P. Expression of genes related to oxidative/nitrosative stress in mouse hearts: Effect of preconditioning and cholesterol diet. *Med Sci Monit*. 2010;16:BR32-39

260. Csont T, Balogh G, Csonka C, Boros I, Horvath I, Vigh L, Ferdinandy P. Hyperlipidemia induced by high cholesterol diet inhibits heat shock response in rat hearts. *Biochem Biophys Res Commun*. 2002;290:1535-1538

261. Wang TD, Chen WJ, Su SS, Lo SC, Lin WW, Lee YT. Increased cardiomyocyte apoptosis following ischemia and reperfusion in diet-induced hypercholesterolemia: Relation to bcl-2 and bax proteins and caspase-3 activity. *Lipids*. 2002;37:385-394

262. Tatsumi T, Matoba S, Kobara M, Keira N, Kawahara A, Tsuruyama K, Tanaka T, Katamura M, Nakagawa C, Ohta B, Yamahara Y, Asayama J, Nakagawa M. Energy metabolism after ischemic preconditioning in streptozotocin-induced diabetic rat hearts. *J Am Coll Cardiol*. 1998;31:707-715

263. Nieszner E, Posa I, Kocsis E, Pogatsa G, Preda I, Koltai MZ. Influence of diabetic state and that of different sulfonylureas on the size of myocardial infarction with and without ischemic preconditioning in rabbits. *Exp Clin Endocrinol Diabetes*. 2002;110:212-218

264. Kersten JR, Schmeling TJ, Orth KG, Pagel PS, Warltier DC. Acute hyperglycemia abolishes ischemic preconditioning in vivo. *Am J Physiol*. 1998;275:H721-725

265. Rana A, Goyal N, Ahlawat A, Jamwal S, Reddy BV, Sharma S. Mechanisms involved in attenuated cardio-protective role of ischemic preconditioning in metabolic disorders. *Perfusion*. 2015;30:94-105

266. del Valle HF, Lascano EC, Negroni JA. Ischemic preconditioning protection against stunning in conscious diabetic sheep: Role of glucose, insulin, sarcolemmal and mitochondrial katp channels. *Cardiovasc Res*. 2002;55:642-659

267. Yadav HN, Singh M, Sharma PL. Involvement of gsk-3beta in attenuation of the cardioprotective effect of ischemic preconditioning in diabetic rat heart. *Mol Cell Biochem*. 2010;343:75-81

268. Gu W, Kehl F, Krolikowski JG, Pagel PS, Warltier DC, Kersten JR. Simvastatin restores ischemic preconditioning in the presence of hyperglycemia through a nitric oxide-mediated mechanism. *Anesthesiology*. 2008;108:634-642

269. Li H, Yao W, Liu Z, Xu A, Huang Y, Ma XL, Irwin MG, Xia Z. Hyperglycemia abrogates ischemic postconditioning cardioprotection by impairing adipor1/caveolin-3/stat3 signaling in diabetic rats. *Diabetes*. 2015

270. Najafi M, Farajnia S, Mohammadi M, Badalzadeh R, Ahmadi Asl N, Baradaran B, Amani M. Inhibition of mitochondrial permeability transition pore restores the cardioprotection by postconditioning in diabetic hearts. *J Diabetes Metab Disord*. 2014;13:106

271. Boutros A, Wang J. Ischemic preconditioning, adenosine and bethanechol protect spontaneously hypertensive isolated rat hearts. *J Pharmacol Exp Ther*. 1995;275:1148-1156

272. Speechly-Dick ME, Baxter GF, Yellon DM. Ischaemic preconditioning protects hypertrophied myocardium. *Cardiovasc Res*. 1994;28:1025-1029

273. Moolman JA, Genade S, Tromp E, Opie LH, Lochner A. Ischaemic preconditioning does not protect hypertrophied myocardium against ischaemia. *S Afr Med J*. 1997;87 Suppl 3:C151-156

274. Penna C, Tullio F, Moro F, Folino A, Merlino A, Pagliaro P. Effects of a protocol of ischemic postconditioning and/or captopril in hearts of normotensive and hypertensive rats. *Basic Res Cardiol*. 2010;105:181-192

275. Wagner C, Ebner B, Tillack D, Strasser RH, Weinbrenner C. Cardioprotection by ischemic postconditioning is abrogated in hypertrophied myocardium of spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 2013;61:35-41

276. Hernandez-Resendiz S, Roldan FJ, Correa F, Martinez-Abundis E, Osorio-Valencia G, Ruiz-de-Jesus O, Alexanderson-Rosas E, Vigueras RM, Franco M, Zazueta C. Postconditioning protects against reperfusion injury in hypertensive dilated cardiomyopathy by activating mek/erk1/2 signaling. *J Card Fail*. 2013;19:135-146

277. Kristiansen SB, Lofgren B, Stottrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT, Botker HE, Flyvbjerg A. Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. *Diabetologia*. 2004;47:1716-1721

278. Bouhidel O, Pons S, Souktani R, Zini R, Berdeaux A, Ghaleb B. Myocardial ischemic postconditioning against ischemia-reperfusion is impaired in ob/ob mice. *Am J Physiol Heart Circ Physiol*. 2008;295:H1580-1586

279. Wagner C, Kloeting I, Strasser RH, Weinbrenner C. Cardioprotection by postconditioning is lost in wokw rats with metabolic syndrome: Role of glycogen synthase kinase 3beta. *J Cardiovasc Pharmacol*. 2008;52:430-437

280. Abete P, Ferrara N, Cioppa A, Ferrara P, Bianco S, Calabrese C, Cacciatore F, Longobardi G, Rengo F. Preconditioning does not prevent postischemic dysfunction in aging heart. *J Am Coll Cardiol*. 1996;27:1777-1786

281. Tani M, Honma Y, Hasegawa H, Tamaki K. Direct activation of mitochondrial k(atp) channels mimics preconditioning but protein kinase c activation is less effective in middle-aged rat hearts. *Cardiovasc Res*. 2001;49:56-68

282. Ebrahim Z, Yellon DM, Baxter GF. Ischemic preconditioning is lost in aging hypertensive rat heart: Independent effects of aging and longstanding hypertension. *Exp Gerontol*. 2007;42:807-814

283. Fenton RA, Dickson EW, Meyer TE, Dobson JG, Jr. Aging reduces the cardioprotective effect of ischemic preconditioning in the rat heart. *J Mol Cell Cardiol*. 2000;32:1371-1375

284. Schulman D, Latchman DS, Yellon DM. Effect of aging on the ability of preconditioning to protect rat hearts from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2001;281:H1630-1636

285. Przyklenk K, Li G, Whittaker P. No loss in the in vivo efficacy of ischemic preconditioning in middle-aged and old rabbits. *J Am Coll Cardiol*. 2001;38:1741-1747

286. Abete P, Ferrara N, Cacciatore F, Sagnelli E, Manzi M, Carnovale V, Calabrese C, de Santis D, Testa G, Longobardi G, Napoli C, Rengo F. High level of physical activity preserves the cardioprotective effect of preinfarction angina in elderly patients. *J Am Coll Cardiol*. 2001;38:1357-1365

287. Burns PG, Krunkenkamp IB, Calderone CA, Kirvaitis RJ, Gaudette GR, Levitsky S. Is the preconditioning response conserved in senescent myocardium? *Ann Thorac Surg*. 1996;61:925-929

288. Przyklenk K, Maynard M, Darling CE, Whittaker P. Aging mouse hearts are refractory to infarct size reduction with post-conditioning. *J Am Coll Cardiol*. 2008;51:1393-1398

289. Huxley RR, Woodward M. Cigarette smoking as a risk factor for coronary heart disease in women compared with men: A systematic review and meta-analysis of prospective cohort studies. *Lancet*. 2011;378:1297-1305

290. Quillen JE, Rossen JD, Oskarsson HJ, Minor RL, Jr., Lopez AG, Winniford MD. Acute effect of cigarette smoking on the coronary circulation: Constriction of epicardial and resistance vessels. *J Am Coll Cardiol*. 1993;22:642-647

291. Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, Deanfield JE. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation*. 1993;88:2149-2155

292. Stefanadis C, Tsiamis E, Vlachopoulos C, Stratos C, Toutouzas K, Pitsavos C, Marakas S, Boudoulas H, Toutouzas P. Unfavorable effect of smoking on the elastic properties of the human aorta. *Circulation*. 1997;95:31-38

293. Nemr R, Lasserre B, Chahine R. Effects of nicotine on thromboxane/prostacyclin balance in myocardial ischemia. *Prostaglandins Leukot Essent Fatty Acids*. 2003;68:191-195

294. Przyklenk K. Nicotine exacerbates postischemic contractile dysfunction of 'stunned' myocardium in the canine model. Possible role of free radicals. *Circulation*. 1994;89:1272-1281

295. Schror K, Zimmermann KC, Tannhauser R. Augmented myocardial ischaemia by nicotine--mechanisms and their possible significance. *Br J Pharmacol*. 1998;125:79-86

296. Zhu BQ, Sun YP, Sievers RE, Glantz SA, Parmley WW, Wolfe CL. Exposure to environmental tobacco smoke increases myocardial infarct size in rats. *Circulation*. 1994;89:1282-1290

297. Talukder MA, Johnson WM, Varadharaj S, Lian J, Kearns PN, El-Mahdy MA, Liu X, Zweier JL. Chronic cigarette smoking causes hypertension, increased oxidative stress, impaired no bioavailability, endothelial dysfunction, and cardiac remodeling in mice. *Am J Physiol Heart Circ Physiol*. 2011;300:H388-396

298. Yamada S, Zhang XQ, Kadono T, Matsuoka N, Rollins D, Badger T, Rodesch CK, Barry WH. Direct toxic effects of aqueous extract of cigarette smoke on cardiac myocytes at clinically relevant concentrations. *Toxicol Appl Pharmacol*. 2009;236:71-77

299. Ménard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. Modulation of l-type calcium channel expression during retinoic acid-induced differentiation of h9c2 cardiac cells. *J Biol Chem*. 1999;274:29063-29070

300. Fulton D, Gratton J-P, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase akt. *Nature*. 1999;399:597-601

301. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by akt-dependent phosphorylation. *Nature*. 1999;399:601-605

302. Michell BJ, Harris MB, Chen Z-p, Ju H, Venema VJ, Blackstone MA, Huang W, Venema RC, Kemp BE. Identification of regulatory sites of phosphorylation of the bovine endothelial nitric-oxide synthase at serine 617 and serine 635. *J Biol Chem*. 2002;277:42344-42351

303. Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B, Schlessinger J. Protein tyrosine kinase pyk2 involved in ca2+-induced regulation of ion channel and map kinase functions. *Nature*. 1995;376:737-745

304. Tokiwa G, Dikic I, Lev S, Schlessinger J. Activation of pyk2 by stress signals and coupling with jnk signaling pathway. *Science* 1996;273:792-794

305. Bayer AL, Ferguson AG, Lucchesi PA, Samarel AM. Pyk2 expression and phosphorylation in neonatal and adult cardiomyocytes. *J Mol Cell Cardiol*. 2001;33:1017-1030

306. Chappell AE, Bunz M, Smoll E, Dong H, Lytle C, Barrett KE, McCole DF. Hydrogen peroxide inhibits ca2+-dependent chloride secretion across colonic epithelial cells via distinct kinase signaling pathways and ion transport proteins. *FASEB J*. 2008;22:2023-2036

307. Loot AE, Schreiber JG, Fisslthaler B, Fleming I. Angiotensin ii impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase. *J Exp Med*. 2009;206:2889-2896

308. Tai L-K, Okuda M, Abe J-i, Yan C, Berk BC. Fluid shear stress activates proline-rich tyrosine kinase via reactive oxygen species–dependent pathway. *Arterioscler Thromb Vasc Biol*. 2002;22:1790-1796

309. Yin G, Yan C, Berk BC. Angiotensin ii signaling pathways mediated by tyrosine kinases. *Int J Biochem Cell Biol*. 2003;35:780-783

310. Melendez J, Welch S, Schaefer E, Moravec CS, Avraham S, Avraham H, Sussman MA. Activation of pyk2/related focal adhesion tyrosine kinase and focal adhesion kinase in cardiac remodeling. *J Biol Chem*. 2002;277:45203-45210

311. Takeishi Y. Pivotal roles of regulating the proline-rich tyrosine kinase 2 (pyk2) signaling in cardiac function and remodeling. *J Mol Cell Cardiol*. 2014;74:295-296

312. Bayer AL, Heidkamp MC, Patel N, Porter MJ, Engman SJ, Samarel AM. Pyk2 expression and phosphorylation increases in pressure overload-induced left ventricular hypertrophy. *Am J Physiol. Heart Circ Physiol*. 2002;283:H695-H706

313. Hirotani S, Higuchi Y, Nishida K, Nakayama H, Yamaguchi O, Hikoso S, Takeda T, Kashiwase K, Watanabe T, Asahi M, Taniike M, Tsujimoto I, Matsumura Y, Sasaki T, Hori M, Otsu K. Ca2+-sensitive tyrosine kinase pyk2/cak β-dependent signaling is essential for g-protein-coupled receptor agonist-induced hypertrophy. *J Cell Mol Cardiol*. 2004;36:799-807

314. Menashi EB, Loftus JC. Differential effects of pyk2 and fak on the hypertrophic response of cardiac myocytes. *Cell Tissue Res*. 2009;337:243-255

315. Koshman YE, Chu M, Kim T, Kalmanson O, Farjah M, Kumar M, Lewis W, Geenen DL, de Tombe P, Goldspink PH, Solaro RJ, Samarel AM. Cardiomyocyte-specific expression of crnk, the c-terminal domain of pyk2, maintains ventricular function and slows ventricular remodeling in a mouse model of dilated cardiomyopathy. *J Mol Cell Cardiol*. 2014;72:281-291

316. Yang C-M, Lee IT, Hsu R-C, Chi P-L, Hsiao L-D. Nadph oxidase/ros-dependent pyk2 activation is involved in tnf-α-induced matrix metalloproteinase-9 expression in rat heart-derived h9c2 cells. *Tox Appl Pharmacol*. 2013;272:431-442

317. Fisslthaler B, Loot AE, Mohamed A, Busse R, Fleming I. Inhibition of endothelial nitric oxide synthase activity by proline-rich tyrosine kinase 2 in response to fluid shear stress and insulin. *Circ Res*. 2008;102:1520-1528

318. Chien PT-Y, Lin C-C, Hsiao L-D, Yang C-M. C-src/pyk2/egfr/pi3k/akt/creb-activated pathway contributes to human cardiomyocyte hypertrophy: Role of cox-2 induction. *Mol Cell Endocrinol*. 2015;409:59-72

319. Matsui A, Okigaki M, Amano K, Adachi Y, Jin D, Takai S, Yamashita T, Kawashima S, Kurihara T, Miyazaki M, Tateishi K, Matsunaga S, Katsume A, Honshou S, Takahashi T, Matoba S, Kusaba T, Tatsumi T, Matsubara H. Central role of calcium-dependent tyrosine kinase pyk2 in endothelial nitric oxide synthase–mediated angiogenic response and vascular function. *Circulation*. 2007;116:1041-1051

320. Sartoretto JL, Kalwa H, Shiroto T, Sartoretto SM, Pluth MD, Lippard SJ, Michel T. Role of ca(2+) in the control of h(2)o(2)-modulated phosphorylation pathways leading to enos activation in cardiac myocytes. *PLoS ONE*. 2012;7:e44627

321. Guo J, Sabri A, Elouardighi H, Rybin V, Steinberg SF. A1-adrenergic receptors activate akt via a pyk2/pdk-1 pathway that is tonically inhibited by novel protein kinase c isoforms in cardiomyocytes. *Circ Res*. 2006;99:1367-1375

322. Loot AE, Schreiber JG, Fisslthaler B, Fleming I. Angiotensin ii impairs endothelial function via tyrosine phosphorylation of the endothelial nitric oxide synthase. *J Exp Med*. 2009;206:2889-2896

323. Edwards DH, Li Y, Griffith TM. Hydrogen peroxide potentiates the edhf phenomenon by promoting endothelial ca2+ mobilization. *Arterioscler Thromb Vasc Biol*. 2008;28:1774-1781

324. Dossumbekova A, Berdyshev EV, Gorshkova I, Shao Z, Li C, Long P, Joshi A, Natarajan V, Vanden Hoek TL. Akt activates nos3 and separately restores barrier integrity in h2o2-stressed human cardiac microvascular endothelium. *Am J Physiol Heart Circ Physiol*. 2008;295:H2417-2426

325. Thomas SR, Chen K, Keaney JF, Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem*. 2002;277:6017-6024

326. Sartoretto JL, Kalwa H, Shiroto T, Sartoretto SM, Pluth MD, Lippard SJ, Michel T. Role of ca2+ in the control of h2o2-modulated phosphorylation pathways leading to enos activation in cardiac myocytes. *PLoS One*. 2012;7:e44627

327. Chouchani Edward T, Pell Victoria R, James Andrew M, Work Lorraine M, Saeb-Parsy K, Frezza C, Krieg T, Murphy Michael P. A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. *Cell Metabol*. 2016

328. Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci USA*. 1987;84:1404-1407

329. Inserte J, Hernando V, Vilardosa Ú, Abad E, Poncelas-Nozal M, Garcia-Dorado D. Activation of cgmp/protein kinase g pathway in postconditioned myocardium depends on reduced oxidative stress and preserved endothelial nitric oxide synthase coupling. *J Am Heart Assoc*. 2013;2:e005975

330. Dumitrescu C, Biondi R, Xia Y, Cardounel AJ, Druhan LJ, Ambrosio G, Zweier JL. Myocardial ischemia results in tetrahydrobiopterin (bh(4)) oxidation with impaired endothelial function ameliorated by bh(4). *Proc Natl Acad Sci USA*. 2007;104:15081-15086

331. Cai M, Li Y, Xu Y, Swartz HM, Chen C-L, Chen Y-R, He G. Endothelial nos activity and myocardial oxygen metabolism define the salvageable ischemic time window for ischemic postconditioning. *Am J Physiol. Heart Circ Physiol*. 2011;300:H1069-H1077

332. Calvert JW, Gundewar S, Jha S, Greer JJM, Bestermann WH, Tian R, Lefer DJ. Acute metformin therapy confers cardioprotection against myocardial infarction via ampk-enos–mediated signaling. *Diabetes*. 2008;57:696-705

333. Giraldez RR, Panda A, Xia Y, Sanders SP, Zweier JL. Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J Biol Chem*. 1997;272:21420-21426

334. Padilla F, Garcia-Dorado D, Agulló L, Barrabés JA, Inserte J, Escalona N, Meyer M, Mirabet M, Pina P, Soler-Soler J. Intravenous administration of the natriuretic peptide urodilatin at low doses during coronary reperfusion limits infarct size in anesthetized pigs. *Cardiovasc Res*. 2001;51:592-600

335. Hamid SA, Totzeck M, Drexhage C, Thompson I, Fowkes RC, Rassaf T, Baxter GF. Nitric oxide/cgmp signalling mediates the cardioprotective action of adrenomedullin in reperfused myocardium. *Basic Res Cardiol*. 2009;105:257-266

336. Inserte J, Barba I, Poncelas-Nozal M, Hernando V, Agulló L, Ruiz-Meana M, Garcia-Dorado D. Cgmp/pkg pathway mediates myocardial postconditioning protection in rat hearts by delaying normalization of intracellular acidosis during reperfusion. *J Mol Cell Cardiol*. 2011;50:903-909

337. Tian Y, Linden J, French BA, Yang Z. Atorvastatin at reperfusion reduces myocardial infarct size in mice by activating enos in bone marrow-derived cells. *PLoS ONE*. 2014;9:e114375

338. Wei H, Vander Heide RS. Heat stress activates akt via focal adhesion kinase-mediated pathway in neonatal rat ventricular myocytes. *Am J Physiol Heart Circ Physiol*. 2008;295:H561-H568

339. Elrod JW, Greer JJM, Bryan NS, Langston W, Szot JF, Gebregzlabher H, Janssens S, Feelisch M, Lefer DJ. Cardiomyocyte-specific overexpression of no synthase-3 protects against myocardial ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol.* 2006;26:1517-1523

340. Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E, Sydow K, Fichtlscherer B, Mulsch A, Munzel T. Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circ Res*. 2003;93:e104-112

341. Abrams J. Hemodynamic effects of nitroglycerin and long-acting nitrates. *Am Heart J*. 1985;110:216-224

342. Flaherty JT. Role of nitroglycerin in acute myocardial infarction. *Cardiology*. 1989;76:122-131

343. Csont T. Nitroglycerin-induced preconditioning: Interaction with nitrate tolerance. *Am J Physiol Heart Circ Physiol*. 2010;298:H308-309

344. Dragoni S, Gori T, Lisi M, Di Stolfo G, Pautz A, Kleinert H, Parker JD. Pentaerythrityl tetranitrate and nitroglycerin, but not isosorbide mononitrate, prevent endothelial dysfunction induced by ischemia and reperfusion. *Arterioscler Thromb Vasc Biol*. 2007;27:1955-1959

345. Bibli SI, Andreadou I, Chatzianastasiou A, Tzimas C, Sanoudou D, Kranias E, Brouckaert P, Coletta C, Szabo C, Kremastinos DT, Iliodromitis EK, Papapetropoulos A. Cardioprotection by h2s engages a cgmp-dependent protein kinase g/phospholamban pathway. *Cardiovasc Res*. 2015;106:432-442

346. Hausenloy DJ, Iliodromitis EK, Andreadou I, Papalois A, Gritsopoulos G, Anastasiou-Nana M, Kremastinos DT, Yellon DM. Investigating the signal transduction pathways underlying remote ischemic conditioning in the porcine heart. *Cardiovasc Drugs Ther*. 2012;26:87-93

347. Wang C, Chiari PC, Weihrauch D, Krolikowski JG, Warltier DC, Kersten JR, Pratt PF, Jr., Pagel PS. Gender-specificity of delayed preconditioning by isoflurane in rabbits: Potential role of endothelial nitric oxide synthase. *Anesth Analg*. 2006;103:274-280, table of contents

348. Guo Y, Stein AB, Wu WJ, Zhu X, Tan W, Li Q, Bolli R. Late preconditioning induced by no donors, adenosine a1 receptor agonists, and delta1-opioid receptor agonists is mediated by inos. *Am J Physiol Heart Circ Physiol*. 2005;289:H2251-2257

349. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, Skatchkov M, Thaiss F, Stahl RA, Warnholtz A, Meinertz T, Griendling K, Harrison DG, Forstermann U, Munzel T. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res*. 2001;88:E14-22

350. Zhao X, Chen YR, He G, Zhang A, Druhan LJ, Strauch AR, Zweier JL. Endothelial nitric oxide synthase (nos3) knockout decreases nos2 induction, limiting hyperoxygenation and conferring protection in the postischemic heart. *Am J Physiol Heart Circ Physiol*. 2007;292:H1541-1550

351. Jugdutt BI. Nitrates in myocardial infarction. *Cardiovasc Drugs Ther*. 1994;8:635-646

352. Jugdutt BI. Intravenous nitroglycerin unloading in acute myocardial infarction. *Am J Cardiol*. 1991;68:52D-63D

353. Jugdutt BI, Warnica JW. Intravenous nitroglycerin therapy to limit myocardial infarct size, expansion, and complications. Effect of timing, dosage, and infarct location. *Circulation*. 1988;78:906-919

354. Bussmann WD, Passek D, Seidel W, Kaltenbach M. Reduction of ck and ck-mb indexes of infarct size by intravenous nitroglycerin. *Circulation*. 1981;63:615-622

355. Garadah T, Ghaisas NK, Mehana N, Foley B, Crean P, Walsh M. Impact of intravenous nitroglycerin on pulsed doppler indexes of left ventricular filling in acute anterior myocardial infarction. *Am Heart J*. 1998;136:812-817

356. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol*. 2004;44:1103-1110

357. Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: Role of coronary flow and pressure and the nitric oxide/cgmp pathway. *Basic Res Cardiol*. 2006;101:168-179

358. Tong G, Aponte AM, Kohr MJ, Steenbergen C, Murphy E, Sun J. Postconditioning leads to an increase in protein s-nitrosylation. *Am J Physiol Heart Circ Physiol*. 2014;306:H825-832

359. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *Br J Pharmacol*. 2003;138:532-543

360. Andreadou I, Iliodromitis EK, Rassaf T, Schulz R, Papapetropoulos A, Ferdinandy P. The role of gasotransmitters no, h2s and co in myocardial ischaemia/reperfusion injury and cardioprotection by preconditioning, postconditioning and remote conditioning. *Br J Pharmacol*. 2015;172:1587-1606

361. Munzel T, Daiber A, Gori T. More answers to the still unresolved question of nitrate tolerance. *Eur Heart J*. 2013;34:2666-2673

362. Jugdutt BI. Use of nitroglycerin for the treatment of acute myocardial infarction. *Heart Vessels*. 1994;9:3-13

363. Prince H. Blood volume in the pregnant rabbit. *Q J Exp Physiol*. 1982;67:87-95

364. Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol*. 2001;21:15-23

365. Mao M, Sudhahar V, Ansenberger-Fricano K, Fernandes DC, Tanaka LY, Fukai T, Laurindo FR, Mason RP, Vasquez-Vivar J, Minshall RD, Stadler K, Bonini MG. Nitroglycerin drives endothelial nitric oxide synthase activation via the phosphatidylinositol 3-kinase/protein kinase b pathway. *Free Radic Biol Med*. 2012;52:427-435

366. Jones SP, Girod WG, Huang PL, Lefer DJ. Myocardial reperfusion injury in neuronal nitric oxide synthase deficient mice. *Coron Artery Dis*. 2000;11:593-597

367. Salloum F, Yin C, Xi L, Kukreja RC. Sildenafil induces delayed preconditioning through inducible nitric oxide synthase-dependent pathway in mouse heart. *Circ Res*. 2003;92:595-597

368. Endres M, Scott G, Namura S, Salzman AL, Huang PL, Moskowitz MA, Szabo C. Role of peroxynitrite and neuronal nitric oxide synthase in the activation of poly(adp-ribose) synthetase in a murine model of cerebral ischemia-reperfusion. *Neurosci Lett*. 1998;248:41-44

369. Bonini MG, Stadler K, Silva SO, Corbett J, Dore M, Petranka J, Fernandes DC, Tanaka LY, Duma D, Laurindo FR, Mason RP. Constitutive nitric oxide synthase activation is a significant route for nitroglycerin-mediated vasodilation. *Proc Natl Acad Sci U S A*. 2008;105:8569-8574

370. Daiber A, Harrison DG, Munzel T. Doubt about an essential role for constitutive nitric oxide synthase in nitroglycerin-mediated vasodilation. *Proc Natl Acad Sci U S A*. 2008;105:E92; author reply E93

371. Schwarz M, Katz SD, Demopoulos L, Hirsch H, Yuen JL, Jondeau G, LeJemtel TH. Enhancement of endothelium-dependent vasodilation by low-dose nitroglycerin in patients with congestive heart failure. *Circulation*. 1994;89:1609-1614

372. Forster C, Main JS, Armstrong PW. Endothelium modulation of the effects of nitroglycerin on blood vessels from dogs with pacing-induced heart failure. *Br J Pharmacol*. 1990;101:109-114

373. Gruhn N, Boesgaard S, Andersen C, Aldershvile J. Nitroglycerin tolerance: Different mechanisms in vascular segments with or without intact endothelial function. *J Cardiovasc Pharmacol*. 2002;40:201-209

374. Parker JO, Parker JD, Caldwell RW, Farrell B, Kaesemeyer WH. The effect of supplemental l-arginine on tolerance development during continuous transdermal nitroglycerin therapy. *J Am Coll Cardiol*. 2002;39:1199-1203

375. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R, Corson MA. Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor ly294002. *J Biol Chem*. 1999;274:30101-30108

376. Abou-Mohamed G, Kaesemeyer WH, Caldwell RB, Caldwell RW. Role of l-arginine in the vascular actions and development of tolerance to nitroglycerin. *Br J Pharmacol*. 2000;130:211-218

377. Levrand S, Vannay-Bouchiche C, Pesse B, Pacher P, Feihl F, Waeber B, Liaudet L. Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo. *Free Radic Biol Med*. 2006;41:886-895

378. Obrosova IG. Peroxynitrite and cardiomyocyte cell death: An evolving story. A commentary on "peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo". *Free Radic Biol Med*. 2006;41:866-868

379. Ohlin H, Pavlidis N, Ohlin AK. Effect of intravenous nitroglycerin on lipid peroxidation after thrombolytic therapy for acute myocardial infarction. *Am J Cardiol*. 1998;82:1463-1467

380. Fekete V, Murlasits Z, Aypar E, Bencsik P, Sarkozy M, Szenasi G, Ferdinandy P, Csont T. Myocardial postconditioning is lost in vascular nitrate tolerance. *J Cardiovasc Pharmacol*. 2013;62:298-303

381. Ullrich V, Schildknecht S. Sensing hypoxia by mitochondria: A unifying hypothesis involving s-nitrosation. *Antioxid Redox Signal*. 2014;20:325-338

382. Nguyen TT, Stevens MV, Kohr M, Steenbergen C, Sack MN, Murphy E. Cysteine 203 of cyclophilin d is critical for cyclophilin d activation of the mitochondrial permeability transition pore. *J Biol Chem*. 2011;286:40184-40192

383. Chen X, Zhang H, McAfee S, Zhang C. The reciprocal relationship between adiponectin and lox-1 in the regulation of endothelial dysfunction in apoe knockout mice. *Am J Physiol Heart Circ Physiol*. 2010;299:H605-612

384. Takaya T, Hirata K, Yamashita T, Shinohara M, Sasaki N, Inoue N, Yada T, Goto M, Fukatsu A, Hayashi T, Alp NJ, Channon KM, Yokoyama M, Kawashima S. A specific role for enos-derived reactive oxygen species in atherosclerosis progression. *Arterioscler Thromb Vasc Biol*. 2007;27:1632-1637

385. Polhemus DJ, Lefer DJ. Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. *Circ Res*. 2014;114:730-737

386. Bian JS, Yong QC, Pan TT, Feng ZN, Ali MY, Zhou S, Moore PK. Role of hydrogen sulfide in the cardioprotection caused by ischemic preconditioning in the rat heart and cardiac myocytes. *J Pharmacol Exp Ther*. 2006;316:670-678

387. Bliksoen M, Kaljusto ML, Vaage J, Stenslokken KO. Effects of hydrogen sulphide on ischaemia-reperfusion injury and ischaemic preconditioning in the isolated, perfused rat heart. *Eur J Cardiothorac Surg*. 2008;34:344-349

388. Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, Kevil CG, Lefer DJ. Hydrogen sulfide mediates cardioprotection through nrf2 signaling. *Circ Res*. 2009;105:365-374

389. Hu LF, Li Y, Neo KL, Yong QC, Lee SW, Tan BK, Bian JS. Hydrogen sulfide regulates na+/h+ exchanger activity via stimulation of phosphoinositide 3-kinase/akt and protein kinase g pathways. *J Pharmacol Exp Ther*. 2011;339:726-735

390. Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK, Bian JS. Cardioprotection induced by hydrogen sulfide preconditioning involves activation of erk and pi3k/akt pathways. *Pflugers Arch*. 2008;455:607-616

391. Yao LL, Huang XW, Wang YG, Cao YX, Zhang CC, Zhu YC. Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing gsk-3beta-dependent opening of mptp. *Am J Physiol Heart Circ Physiol*. 2010;298:H1310-1319

392. Yong QC, Lee SW, Foo CS, Neo KL, Chen X, Bian JS. Endogenous hydrogen sulphide mediates the cardioprotection induced by ischemic postconditioning. *Am J Physiol Heart Circ Physiol*. 2008;295:H1330-H1340

393. Koika V, Zhou Z, Vasileiadis I, Roussos C, Finetti F, Monti M, Morbidelli L, Papapetropoulos A. Pkg-i inhibition attenuates vascular endothelial growth factor-stimulated angiogenesis. *Vascul Pharmacol*. 2010;53:215-222

394. Andreadou I, Iliodromitis EK, Tsovolas K, Aggeli IK, Zoga A, Gaitanaki C, Paraskevaidis IA, Markantonis SL, Beis I, Kremastinos DT. Acute administration of vitamin e triggers preconditioning via k(atp) channels and cyclic-gmp without inhibiting lipid peroxidation. *Free Radic Biol Med*. 2006;41:1092-1099

395. Pattison JS, Waggoner JR, James J, Martin L, Gulick J, Osinska H, Klevitsky R, Kranias EG, Robbins J. Phospholamban overexpression in transgenic rabbits. *Transgenic Res*. 2008;17:157-170

396. Predmore BL, Kondo K, Bhushan S, Zlatopolsky MA, King AL, Aragon JP, Grinsfelder DB, Condit ME, Lefer DJ. The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. *Am J Physiol Heart Circ Physiol*. 2012;302:H2410-2418

397. Inserte J, Hernando V, Garcia-Dorado D. Contribution of calpains to myocardial ischaemia/reperfusion injury. *Cardiovasc Res*. 2012;96:23-31

398. Bucci M, Papapetropoulos A, Vellecco V, Zhou Z, Zaid A, Giannogonas P, Cantalupo A, Dhayade S, Karalis KP, Wang R, Feil R, Cirino G. Cgmp-dependent protein kinase contributes to hydrogen sulfide-stimulated vasorelaxation. *PLoS One*. 2012;7:e53319

399. Wang R. Physiological implications of hydrogen sulfide: A whiff exploration that blossomed. *Physiol Rev*. 2012;92:791-896

400. Brown DA, Chicco AJ, Jew KN, Johnson MS, Lynch JM, Watson PA, Moore RL. Cardioprotection afforded by chronic exercise is mediated by the sarcolemmal, and not the mitochondrial, isoform of the katp channel in the rat. *J Physiol*. 2005;569:913-924

401. Luo W, Grupp IL, Harrer J, Ponniah S, Grupp G, Duffy JJ, Doetschman T, Kranias EG. Targeted ablation of the phospholamban gene is associated with markedly enhanced myocardial contractility and loss of beta-agonist stimulation. *Circ Res*. 1994;75:401-409

402. Chu G, Kerr JP, Mitton B, Egnaczyk GF, Vazquez JA, Shen M, Kilby GW, Stevenson TI, Maggio JE, Vockley J, Rapundalo ST, Kranias EG. Proteomic analysis of hyperdynamic mouse hearts with enhanced sarcoplasmic reticulum calcium cycling. *FASEB J*. 2004;18:1725-1727

403. Murgia M, Giorgi C, Pinton P, Rizzuto R. Controlling metabolism and cell death: At the heart of mitochondrial calcium signalling. *J Mol Cell Cardiol*. 2009;46:781-788

404. Pan TT, Neo KL, Hu LF, Yong QC, Bian JS. H2s preconditioning-induced pkc activation regulates intracellular calcium handling in rat cardiomyocytes. *Am J Physiol Cell Physiol*. 2008;294:C169-177

405. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabo C. Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Natl Acad Sci U S A*. 2009;106:21972-21977

406. Cohen JI, Chen X, Nagy LE. Redox signaling and the innate immune system in alcoholic liver disease. *Antioxid Redox Signal*. 2011;15:523-534

407. Heusch G, Skyschally A, Schulz R. The in-situ pig heart with regional ischemia/reperfusion - ready for translation. *J Mol Cell Cardiol*. 2011;50:951-963

408. Minamishima S, Bougaki M, Sips PY, Yu JD, Minamishima YA, Elrod JW, Lefer DJ, Bloch KD, Ichinose F. Hydrogen sulfide improves survival after cardiac arrest and cardiopulmonary resuscitation via a nitric oxide synthase 3-dependent mechanism in mice. *Circulation*. 2009;120:888-896

409. Kondo K, Bhushan S, King AL, Prabhu SD, Hamid T, Koenig S, Murohara T, Predmore BL, Gojon G, Sr., Gojon G, Jr., Wang R, Karusula N, Nicholson CK, Calvert JW, Lefer DJ. H(2)s protects against pressure overload-induced heart failure via upregulation of endothelial nitric oxide synthase. *Circulation*. 2013;127:1116-1127

410. Messner B, Bernhard D. Smoking and cardiovascular disease: Mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol*. 2014;34:509-515

411. D'Hulst A I, Maes T, Bracke KR, Demedts IK, Tournoy KG, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir Res*. 2005;6:147

412. Andreadou I, Mikros E, Ioannidis K, Sigala F, Naka K, Kostidis S, Farmakis D, Tenta R, Kavantzas N, Bibli SI, Gikas E, Skaltsounis L, Kremastinos DT, Iliodromitis EK. Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. *J Mol Cell Cardiol*. 2014;69:4-16

413. Guo X, Oldham MJ, Kleinman MT, Phalen RF, Kassab GS. Effect of cigarette smoking on nitric oxide, structural, and mechanical properties of mouse arteries. *Am J Physiol Heart Circ Physiol*. 2006;291:H2354-2361

414. Lisi M, Dragoni S, Leone MC, Munzel T, Parker JD, Gori T. Acute (but not chronic) smoking paradoxically protects the endothelium from ischemia and reperfusion: Insight into the "smoking paradox". *Clin Res Cardiol*. 2013;102:387-389

415. Barua RS, Ambrose JA, Srivastava S, DeVoe MC, Eales-Reynolds LJ. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase: An in vitro demonstration in human coronary artery endothelial cells. *Circulation*. 2003;107:2342-2347

416. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial no synthesis by multi-site enos phosphorylation. *J Mol Cell Cardiol*. 2007;42:271-279

417. Qian J, Fulton D. Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. *Front Physiol*. 2013;4:347

418. Arunachalam G, Yao H, Sundar IK, Caito S, Rahman I. Sirt1 regulates oxidant- and cigarette smoke-induced enos acetylation in endothelial cells: Role of resveratrol. *Biochem Biophys Res Commun*. 2010;393:66-72

419. Liu L, Shang F, Morgan MJ, King DW, Lubowski DZ, Burcher E. Cyclooxygenase-dependent alterations in substance p-mediated contractility and tachykinin nk1 receptor expression in the colonic circular muscle of patients with slow transit constipation. *J Pharmacol Exp Ther*. 2009;329:282-289

420. Zhou X, Zhao L, Mao J, Huang J, Chen J. Antioxidant effects of hydrogen sulfide on left ventricular remodeling in smoking rats are mediated via pi3k/akt-dependent activation of nrf2. *Toxicol Sci*. 2015;144:197-203

421. Meng G, Ma Y, Xie L, Ferro A, Ji Y. Emerging role of hydrogen sulfide in hypertension and related cardiovascular diseases. *Br J Pharmacol*. 2015;172:5501-5511

422. Fantinelli JC, Mosca SM. Comparative effects of ischemic pre and postconditioning on ischemia-reperfusion injury in spontaneously hypertensive rats (shr). *Mol Cell Biochem*. 2007;296:45-51

423. Penna C, Tullio F, Perrelli MG, Moro F, Abbadessa G, Piccione F, Carriero V, Racca S, Pagliaro P. Ischemia/reperfusion injury is increased and cardioprotection by a postconditioning protocol is lost as cardiac hypertrophy develops in nandrolone treated rats. *Basic Res Cardiol*. 2011;106:409-420

424. Wagner L, Laczy B, Tamasko M, Mazak I, Marko L, Molnar GA, Wagner Z, Mohas M, Cseh J, Fekete A, Wittmann I. Cigarette smoke-induced alterations in endothelial nitric oxide synthase phosphorylation: Role of protein kinase c. *Endothelium*. 2007;14:245-255

425. Cohen MV, Downey JM. Signalling pathways and mechanisms of protection in pre- and postconditioning: Historical perspective and lessons for the future. *Br J Pharmacol*. 2015;172:1913-1932

426. Barsukevich V, Basalay M, Sanchez J, Mrochek A, Whittle J, Ackland GL, Gourine AV, Gourine A. Distinct cardioprotective mechanisms of immediate, early and delayed ischaemic postconditioning. *Basic Res Cardiol*. 2015;110:452

427. Hausenloy DJ, Yellon DM. The therapeutic potential of ischemic conditioning: An update. *Nat Rev Cardiol*. 2011;8:619-629

428. Penna C, Mancardi D, Rastaldo R, Pagliaro P. Cardioprotection: A radical view free radicals in pre and postconditioning. *Biochim Biophys Acta*. 2009;1787:781-793

429. Iliodromitis EK, Cohen MV, Dagres N, Andreadou I, Kremastinos DT, Downey JM. What is wrong with cardiac conditioning? We may be shooting at moving targets. *J Cardiovasc Pharmacol Ther*. 2015;20:357-369

430. Hozawa A, Okamura T, Murakami Y, Kadowaki T, Nakamura K, Hayakawa T, Kita Y, Nakamura Y, Abbott RD, Okayama A, Ueshima H. Joint impact of smoking and hypertension on cardiovascular disease and all-cause mortality in japan: Nippon data80, a 19-year follow-up. *Hypertens Res*. 2007;30:1169-1175

431. Sloth AD, Schmidt MR, Munk K, Schmidt M, Pedersen L, Sorensen HT, Botker HE. Impact of cardiovascular risk factors and medication use on the efficacy of remote ischaemic

conditioning: Post hoc subgroup analysis of a randomised controlled trial. *BMJ Open*. 2015;5:e006923