ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

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ΝΟΣΟΚΟΜΕΙΟ ΛΑΪΚΟ

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ΑΝΑΠΛΗΡΩΤΗΣ ΚΑΘΗΓΗΤΗΣ ΕΚΠΑ

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ΑΘΗΝΑ 2018

Τα μέλη της Τριμελούς Εξεταστικής Επιτροπής:

Καθηγητής Νικόλαος Τεντολούρης

Αναπληρωτής Καθηγητής Κωνσταντίνος Μακρυλάκης

Αναπληρωτής Καθηγητής Αλέξανδρος Κόκκινος

Ημερομηνία εξέτασης: 20 Ιουνίου 2018

Τίτλος: “ Η επίδραση της από του στόματος χορήγησης λευκίνης στην ενδοθηλιο - εξαρτώμενη αγγειοδιαστολή κατά την οξεία υπεργλυκαιμία σε υγιή άτομα”.

Title: “The effect of the oral administration of leucine on endothelial function, glucose and insulin concentrations in healthy subjects**”.**

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Exp Clin Endocrinol Diabetes. 2018 Jun 11.

Στις κόρες μου Χλόη και Λουίζα

Επιθυμώ να ευχαριστήσω:

Τον **Καθηγητή Νικόλαο Κατσιλάμπρο**, ο οποίος ενεπνεύστη το θέμα αυτής της ερευνητικής εργασίας και υπήρξε ταυτόχρονα ο κύριος δάσκαλός μου σε όλα τα έτη της ενασχόλησής μου με το Σακχαρώδη Διαβήτη.

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ABSTRACT

Objective: The aim of our study was to investigate the potential differential effect of hyperglycaemia and hyperinsulinaemia induced by glucose infusion alone and in combination with leucine consumption on endothelial function in healthy individuals.

Methods: Ten male volunteers were examined in random order twice. In one visit, they consumed 250ml water (baseline) and 30 min later glucose was infused iv. In the other visit, they consumed 250ml water with 25g of leucine and 30 min later the same amount of glucose was infused. Serum glucose and insulin were measured at baseline and every 10 min after glucose infusion for 1 hour. Endothelial function was evaluated by measurement of flow mediated vasodilatation (FMD) at baseline, 10 and 60 min after glucose infusion.

Results: In both visits, glucose levels increased to the same degree, whereas insulin response was significantly higher after leucine administration. FMD values declined significantly compared to baseline 10 min after glucose infusion in the control visit (6.9 ±2.7 vs. 3.2 ±3.5%, respectively, p=0.006), while no significant change was observed when glucose infusion was followed by leucine consumption.

Conclusions: Acute hyperglycaemia impairs endothelial function in healthy male individuals. Leucine administration prevents hyperglycaemia-mediated endothelial dysfunction probably due to enhanced insulin secretion.

List of Abbreviations

BMI: body mass index

FMD: flow-mediated dilatation

ANOVA: analysis of variance

AUC: area under the curve

AGEs: advanced glycation end-products

PKC: activation of protein kinace C

ROS: reactive oxygen species

1. INTRODUCTION

Hyperglycemia is a well known factor of atherothrombotic disease, and can impair endothelial function in both diabetic and non diabetic individuals. It significantly contributes to the development of both cardiovascular and microvascular complications of Type 2 diabetes [1] and is considered an independent risk factor for future cardiovascular events [2].

Both chronic and acute hyperglycaemia have detrimental effects on endothelial function [3,4]. There is accumulating evidence that postprandial hyperglycemia is an independent risk factor for atherosclerosis [5] and cardiovascular disease and has effects greater than that of fasting hyperglycemia [6,7].

Atherosclerosis is a progressive disease characterized by the response of the endothelium to chronic inflammation leading to the formation of atheromatous or fibrous plaques. Endothelial dysfunction is considered to be the initial stage of atherosclerosis. In addition to endothelial dysfunction, smooth muscle cell dysfunction metabolic abnormalities of the vessel wall including inflammation, oxidative stress and disruption of neurohormonal balance take place in the early phase of the atherosclerosis process. [8]

Postprandial hyperglycemia is characterized by hyperglycemic peaks that induce endothelial dysfunction, inflammation and oxidative stress, which in turn lead to atherosclerosis, making postprandial hyperglycaemia an important factor in the development of cardiovascular disease [9]. In accordance to the effects of chronic hyperglycemia on the endothelium, acute or postprandial hyperglycemia not only deteriorates vascular endothelial dysfunction in individuals with

chronic hyperglycemia but also transiently impairs endothelium function even in healthy individuals [10]. Postprandial hyperglycemia has been shown to better predict future cardiovascular mortality compared with fasting glucose in both diabetic and non diabetic individuals [10] and is considered an independent predictor of cardiovascular events [10]. Postprandial hyperglycemia seems to harm the vascular endothelium via oxidative stress-mediated imbalance in nitric oxide homeostasis [10]. Post-challenge glucose levels measured during an oral glucose tolerance test could be used as a predictor of postprandial hyperglycaemia [11].

In human studies, patients with diabetes mellitus type I and II are both associated with impaired vascular function [12-18]. Vascular dysfunction is also a well-recognized feature of obesity and insulin resistance even without concurrent hyperglycemia [19,20]. Several studies have even suggested that vascular function is not further impaired by the additional effects of diabetes beyond those of obesity [21].

Detrimental effects of short-term hyperglycemia on peripheral vascular function have been demonstrated in some but not all studies of healthy normoglycemic humans [22,23,24]. In vitro hyperglycemia induces the production of highly reactive oxidant species and induces the production of vasoconstrictors including endothelin-1 [25, 26]. Additionally, there are studies that have showed impaired endothelium function in first-degree relatives of type 2 diabetes mellitus patients in the fasting state as well as during acute hyperglycemia induced by an oral glucose test [27]

Studies evaluating the in vivo effect of acute hyperglycemia on human vascular function provide evidence of glucose-induced impairment in vascular function but also provide evidence showing opposite results [23,24].

In a study by Natali et al. participants with normal glucose tolerance, impaired glucose tolerance and diabetic individuals, were examined. The authors found that

after the oral glucose tolerance test plasma antioxidants levels were significantly reduced by 5% to 10% in all patient groups but the forearm vascular response (plethysmography) to intra-arterial acetylcholine (ACh) infusion was not affected by glucose ingestion in any group, while the response to SNP infusion was attenuated, particularly in the IGT group, concluding that regardless of glucose tolerance, oral glucose does not impair endothelium-dependent vasodilatation in the microcirculation, despite causing increased oxidative stress. The authors attributed the aforementioned finding to the endogenous insulin response that is probably responsible for countering any inhibitory effect on vascular function [23]. Reed et al investigated whether transient hyperglycemia alters vascular function in healthy humans. They measured vasodilator responses in 25 healthy volunteers exposed to one of three glucose levels i.e. 1) glucose mimicking a postprandial pattern observed in individuals with impaired glucose tolerance, 2) 6 h of mild hyperglycemia and 3) 6 h of normal glucose values. Peak endothelium-dependent vasodilator responses to ACh were not diminished by hyperglycemia in any trial. Therefore, the authors concluded that acute moderate hyperglycemia did not impair vascular function. [24]

Another study that included prediabetic individuals showed that the presence of prediabetes (impaired fasting glucose and/or impaired glucose tolerance) was associated with impaired endothelium-dependent vasodilation in non-obese subjects. Vascular function in dysglycemic non-obese subjects was reduced compared to normoglycemic obese subjects. Within the obese subjects group, dysglycemia was not associated with further worsening of the vascular response and while obesity, insulin resistance and dysglycemia were each significantly inversely related to vascular function, multivariable modeling analyses revealed that effects of glycemia were not significant when entered in models together with measures of obesity and

insulin resistance [28]. Moreover, in a study by Kim et al, where the effect of acute hyperglycemia on endothelium-dependent vasodilation in patients with DM or impaired glucose metabolism in vivo was examined by plethysmography, the induction of hyperglycemia resulted in a significant attenuation of endothelial function, implicating the importance of hyperglycemia in the development of endothelial dysfunction commonly observed in patients with diabetes mellitus or impaired glucose metabolism [29]. The impact of acute hyperglycaemia on endothelial function and retinal vascular reactivity was also studied by Chittari et al. [30]. Endothelial function was evaluated by measuring flow-mediated vasodilation of the brachial artery, while retinal vascular reactivity was measured using a retinal vessel analyser, during a flicker. They showed that in basal, fasting conditions, both flow-mediated dilatation and retinal vessel reactivity are decreased in patients with diabetes mellitus type 2 compared with control subjects and furthermore an acute increase of glycaemia generated by an oral glucose tolerance test simultaneously induces an alteration at the level of macro-circulation, and in the retina in patients with diabetes mellitus type 2 [30]. It has been suggested that, as previously mentioned, nitric oxide production is involved both in the effect of hyperglycaemia at the level of forearm circulation as well as at the level of the retinal circulation [31] and furthermore narrowing of the retinal arterioles has been associated with stroke, coronary artery disease and hypertension, independent of other risk factors [32].

Flow-mediated vasodilation was also inversely related to HbA1c but only in non-obese subjects [33]. Elevated HbA1c or elevated body mass index was associated with impaired vascular function but without further impairment seen in subjects with both factors elevated [33]. In the study by Han et al. the contribution of insulin resistance measures was found weaker than that of obesity [28] taking into

consideration as well that insulin resistance mediates the effects of obesity on the endothelium. It is also interesting that several medications commonly prescribed in patients with diabetes mellitus, such as ACE inhibitors, AT-1 blockers and statins can interfere with nitric oxide action [20,35-38].

As previously stated, hyperglycemia is the hallmark of diabetes and there are data linking hyperglycemia to endothelial dysfunction and accelerated atherogenesis [35-38] Moreover, endothelial dysfunction seems to precede plaque formation in atherosclerosis [35-38] Endothelial cell dysfunction is characterized by enhanced permeability, increased cell adhesion molecules, chemokines, cytokines, glycocalyx dysfunction and reduced antithrombotic properties. It is well established that hyperglycemia can induce endothelial cell dysfunction through multiple pathways including the increased oxidative stress (ROS), polyol pathway, hexosamine pathway, activation of protein kinase-C (PKC), advanced glycation end product (AGE) formation, and increased inflammation [35-38]. Inflammation appears to be an important factor in all phases of atherosclerosis from the early lesion to the phase of plaque rupture.

Toll-like receptors are responsible for activating signaling pathways that lead to inflammatory responses in the host cells . Toll-like receptors are increased in patients with diabetes mellitus and play a significant role in the process of atherosclerosis. Additionally, there is a role of Toll-like receptors expression and activation of their signaling pathway in hyperglycemia-induced cell damage [39].

There are conflicting results of the in vivo effect of insulin in the endothelium. Insulin has been traditionally considered to exert vasodilatatory effects through the stimulation of nitric oxide synthesis [40,41]. However, chronic hyperinsulinaemia, an accompanying feature of obesity and insulin resistance, as well as modest prolonged

hyperinsulinaemia similar to that observed in insulin resistant states have been found to be associated with endothelial dysfunction [41,42]. Acute hyperinsulinaemia has been reported to impair macrovascular endothelial function in some studies [43], whereas others have found that hyperinsulinaemia prevents hyperglycaemia-induced endothelial dysfunction [44]. Although the effect of hyperglycaemia on endothelial function cannot be easily separated from the effect of hyperinsulinaemia, since the two usually accompany one another in most clinical situations, data regarding the combined effect of both acute hyperglycaemia and hyperinsulinaemia on endothelial function in healthy individuals are scarce. In an elegant study by Perkins et al the separate and combined effects of hyperglycemia and hyperinsulinemia on markers of endothelial function (proinflammatory and proatherothrombotic responses in overweight/obese nondiabetic humans were studied in 22 individuals in four different pancreatic clamp techniques (4-h glucose clamps) consisting of either euinsulinemia-euglycemia, euinsulinemia-hyperglycemia, hyperinsulinemia-hyperglycemia, or hyperinsulinemia-euglycemia. Various molecules were measured and the authors found that VCAM, ICAM, P-selectin, E-selectin, IL-6, adiponectin, and PAI-1 responses were all increased while endothelial function was decreased during euinsulinemia-hyperglycemia compared with other protocols. In this study, hyperinsulinemia in the presence of hyperglycemia prevented the increase in proinflammatory and proatherothrombotic markers [45].

Branched-chain amino acids, including leucine, isoleucine and valine, are essential amino acids that can stimulate insulin secretion to varying degrees [46]. Several clinical studies have shown that addition of amino acids in a carbohydrate meal results in higher insulin secretion compared to ingestion of carbohydrates alone in both healthy individuals and patients with type 2 diabetes mellitus [47-49].

Regarding leucine, in vitro studies have reported that it enhances insulin secretion from pancreatic β-cells through several mechanisms, while in vivo studies confirmed that leucine administration acutely elevates insulin secretion in both humans and rodents and thus plays an important role in glucose homeostasis [50].

In vivo vascular function measured in peripheral vessels is currently used as a marker of cardiovascular disease status and brachial FMD has been found to be inversely associated with future cardiovascular events [51]. Endothelium exerts an important role in the maintenance of vascular tone and structure. When diminished production of nitric oxide takes place in response to inflammatory stimuli, local vasodilator properties such as reactive hyperemia and increased shear stress are decreased. The degree of this vasodilator response to increased flow i.e. flow mediated dilation (FMD) has been widely used as a surrogate marker of endothelial dysfunction in patients with asymptomatic atherosclerosis, coronary artery disease congestive heart failure (CHF) and other clinical entities [52].

3. **AIM OF THE PRESENT STUDY**

The aim of the present study was to investigate whether acute hyperglycaemia through the intravenous infusion of glucose in healthy subjects undergoing an exaggerated insulin response induced by previous administration of leucine might have a favorable effect on endothelial function.

4. MATERIALS AND METHODS

Ten healthy volunteers were included in this cross-over study. In order to avoid the potential confounding effect of sex hormones on endothelial function, only male individuals were studied [53]. Since the study aimed at assessing the effect of hyperglycemia and leucine administration on endothelial function in healthy conditions, we tested our hypothesis in individuals who could be considered as healthy as possible, and therefore we excluded subjects with any factor that could confer cardiovascular risk. Thus, inclusion criteria required that the participants were non-obese (BMI <30 kg/m^2), non-smokers and without conditions that are associated with endothelial dysfunction, such as clinically apparent macrovascular disease, hypertension, lipid disorders or lipid-lowering medications, diabetes or a family history of type 2 diabetes [54].

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethics Committee of our Hospital. The nature and details of the study were clearly explained and written informed consent was obtained from all participants.

4.1 Study design

All volunteers underwent two study sessions, in a cross-over design and in random order, with an interval of about one week in between. On each study day they arrived at 08:00-08:30 am at the Metabolic Unit of our Department after a 12 hour fast. All tests were performed in a quiet room of constant temperature (22-24°C). Body weight, height, and waist circumference were measured using standard methods and BMI was calculated.

A venous cannula for blood sampling was placed in a superficial forearm vein of the left upper limb and kept patent by bolus 0.9% saline water infusions. In the control visit participants consumed 250 ml of water (baseline, time= -30) and 30 min later 0.5 g/kg of glucose in the form of a 35% solution were infused intravenously (time= 0). In the other visit (leucine visit), participants consumed 25 g of leucine diluted in 250 ml of water at baseline (time= -30). Thirty

min later the same amount of glucose was infused intravenously (time= 0). The 30-min interval was used for the sufficient absorption of leucine.

Blood was drawn before the consumption of water or leucine (baseline, time= -30 min) and every 10 min after glucose infusion throughout the experiment, namely at 10, 20, 30, 40, 50 and 60 min. In the leucine arm of the study, blood was also drawn at time point 0 min, in order to assess the possible hyperinsulinemic effect of leucine.

Samples were immediately centrifuged and serum glucose was measured immediately on an automatic analyser (RA-XT, Doublin, Ireland using the glucose oxidase-peroxidase method), while serum aliquots were promptly frozen at -80°C for the later assessment of insulin concentrations by radioimmunoassay (Biosure, Belgium, c.v. = 3.3 ± 1.2 %).

4.2 FMD measurement

Endothelial function was evaluated by the measurement of FMD of the brachial artery at baseline (time= -30 min), at 10 min and at 60 min after glucose infusion.

Endothelium-dependent FMD in response to reactive hyperaemia was evaluated in the supine position on the brachial artery using high resolution ultrasound and after the individuals had remained supine for at least 20 min [55]. Longitudal images of the brachial artery were measured on B mode ultrasound images by the use of a 7.5 MHZ linear transducer and a high resolution KretzTecknik (KretzTechlink, Austria) ultrasound system. FMD was assessed by measuring the % change of the brachial artery diameter during reactive hyperaemia from baseline. To create this stimulus, a pneumatic cuff was placed 15 cm above the wrist and inflated to 100 mmHg above the systolic blood pressure of the study subjects for 4 min. The brachial artery was then imaged for 30 sec before and between 30 and 90 sec after cuff deflation, while reactive hyperaemia ensued, as previously described [55]. Within this time period, systolic and diastolic arterial blood pressure, heart rate and diameter of the brachial artery were measured. The same experienced observer, who was blinded to the phase of the study, performed all FMD studies.

4.3 Statistical Methods

Statistical analyses were performed using the SPSS 17.0 statistical package (SPSS, Chicago, IL, USA). ANOVA for repeated measurements was performed to assess the effect of water or leucine consumption along with glucose infusion on serum glucose and insulin concentrations over time. The Greenhouse-Geisser adjustment was used when the sphericity assumptions were not fulfilled. A paired *t*-test was performed to compare the values of serum glucose, serum insulin concentrations and FMD values between baseline status and at the examined time intervals during each visit the study, as well as between the two visits of the study at the examined time intervals. The overall response of serum glucose and insulin were calculated as AUC using the trapezoid rule and divided by the total time of the study session (90 min). Statistical significance was assumed at $p<0.05$.

5. RESULTS

The mean age of the participants was 31.8 ± 5.5 years, mean BMI was 26.4 ± 2.7 kg/m^2 and mean waist circumference was 91.3 ± 5.9 cm. All subjects were normotensive: mean systolic blood pressure was 123.3 ± 8.8 mmHg and mean diastolic blood pressure was 73.9 ± 7.0 mmHg.

In the fasting state, neither serum glucose nor insulin concentrations were significantly different between the two visits of the study (Table1). At time point 0 min, i.e. 30 minutes after leucine ingestion, non-statistically significant changes in both glucose and insulin levels were observed vs time point -30 min, although there was a trend towards higher insulin levels (Glucose -30 min: 89 ± 12.3 vs 0 min: 86 ± 10.8 mg/dl, p=0.126, insulin -30 min: 13.6 ± 7.6 vs 17.2 ± 7.3 μu/ml, p=0,085).

A significant increase in serum glucose levels was observed after glucose infusion in both study sessions and remained so until 40 min of the experiment (p values for the effect of time <0.001 at both visits) (Table 1, Figure 1). The peak value was observed at 10 min; afterwards the values declined gradually and returned to baseline at 60 min (Table 1).

No significant differences in serum glucose concentrations were observed at any time point between the two visits, with the exception of the 60 min measurement, at which serum glucose levels were lower in the leucine visit in comparison with the control visit (85.1 ± 31.4 vs 100.7 ± 31.1 mg/dl, respectively, p=0.05). However, the magnitude of the increase in serum glucose concentrations, expressed as AUC, was not significantly different between the two study visits (121.5 ± 30.3 vs. 125.6 ± 19.8 mg·min/dl, respectively, p=0.64).

A significant increase in serum insulin levels was observed after glucose infusion in comparison with baseline values until the end of the experiment in both visits (p values for the effect of time <0.001 at both visits) (Table 1, Figure 1). The peak insulin values were observed at 20 min in both visits; afterwards, they declined gradually until 60 min (Table 1).

Serum insulin concentrations were significantly higher after leucine consumption in comparison with water consumption at all examined time points (all p values <0.01). In

addition, the magnitude of the increase in serum insulin concentrations, expressed as AUC, was significantly higher in the leucine visit in comparison with the control visit (69.5 ± 27.5 vs. 28.4 ± 10.7 μU·min/ml, respectively, $p<0.001$).

Baseline FMD values were not different between the two study visits (6.9 ± 2.7% vs. 6.3 ± 2.9%, respectively, $p=0.572$), highlighting the low day-to-day variation we observed while implementing this method. Mean FMD values declined significantly in comparison with baseline 10 min after glucose infusion in the control visit (6.9 ± 2.7% vs. 3.2 ± 3.5%, respectively, $p=0.006$). On the contrary, no significant change was observed after glucose infusion in the leucine visit (6.3 ± 2.9% vs. 7.1 ± 3.5%, respectively, $p=0.79$). As a consequence, FMD values 10 min after glucose infusion were significantly lower in the control visit when compared with the FMD values at the same time interval in the leucine visit ($p=0.02$). In both visits, FMD values returned to baseline values at the end of the experiment (Figure 3).

Baseline brachial artery diameters (diameter before cuff inflation) at time points 30 min before, and 10 and 60 min after glucose infusion in both arms of the study were not statistically different.

No adverse effects were observed after ingestion of leucine or after glucose infusion.

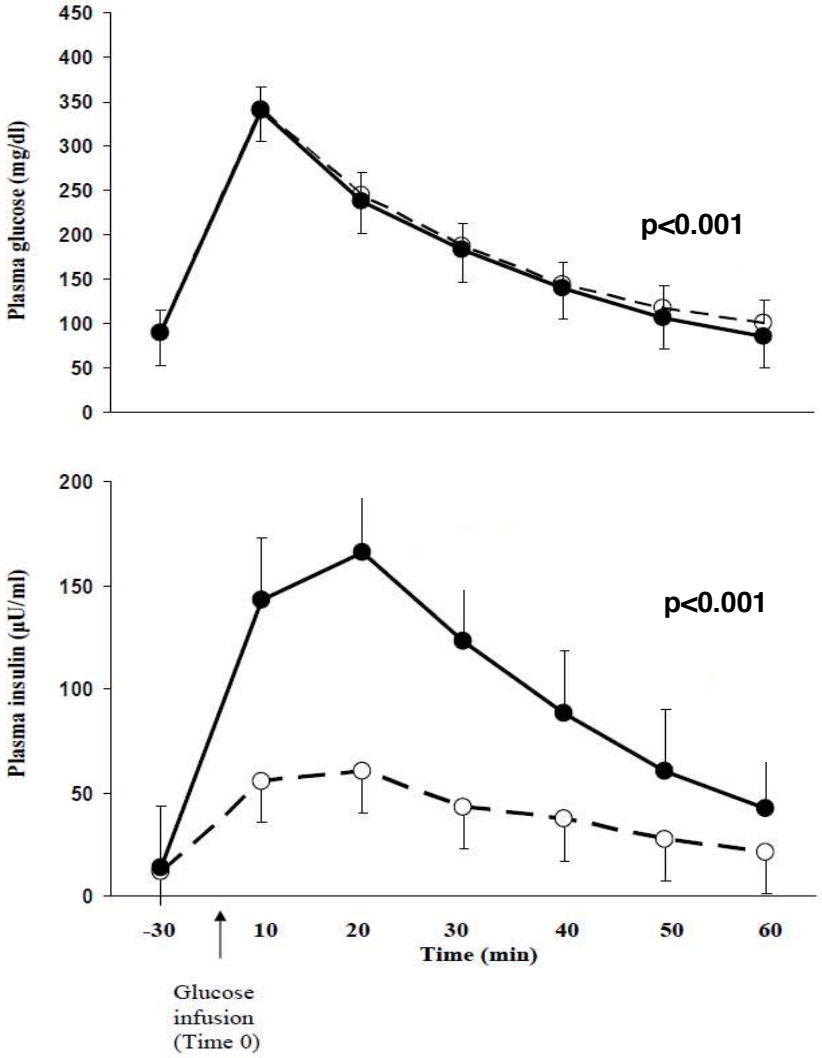
Plasma glucose (mg/dl)
450
400
350
300
250
200
150
100
50
0
p<0.001
Plasma insulin (µU/ml)
200
150
100
50
0
p<0.001
-30
10
20
30
40
50
60
Time (min)
Glucose infusion (Time 0)

Figure 1

Plasma concentrations of glucose (upper panel) and insulin (lower panel) during the two visits of the study.

Continuous lines: leucine consumption and glucose infusion.

Dashed lines: water consumption and glucose infusion.

p values for the effect of water or leucine consumption over time by ANOVA for repeated measurements.

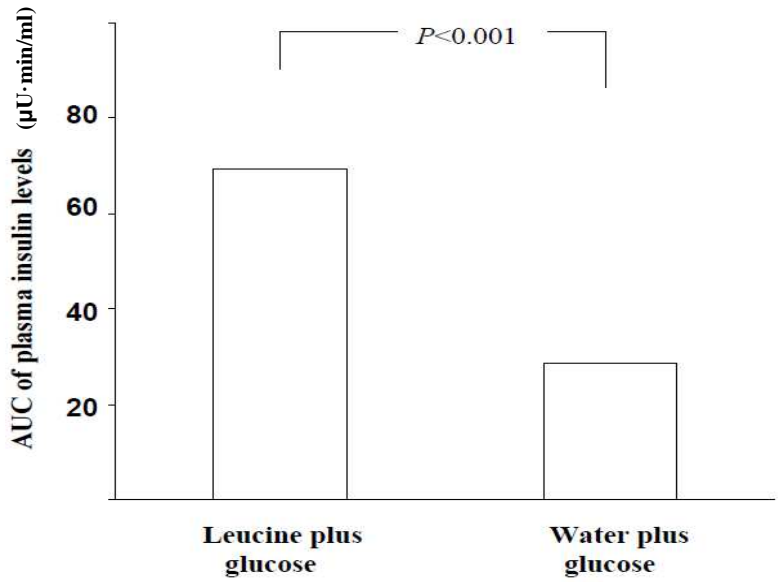

Figure 2

Plasma insulin responses during the two visits of the study expressed as area under the curve (AUC) and divided by the time observation (90 min).

p value for comparison between the two visits of the study by paired t-test.

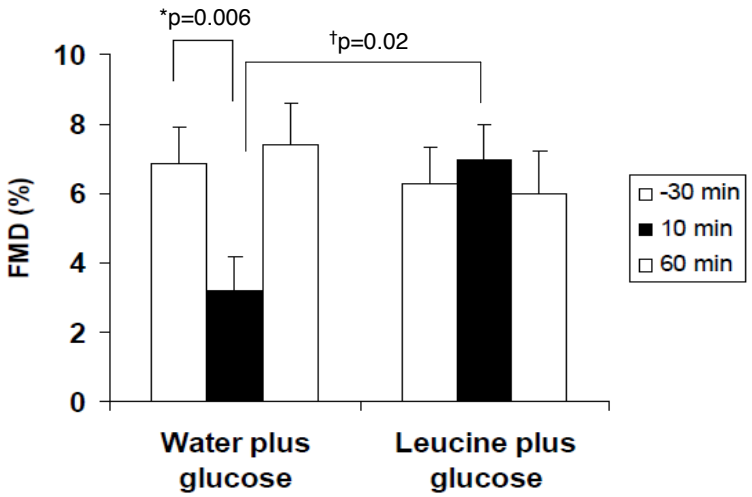

Figure 3

Flow-mediated dilatation during the two visits of the study.

*p value for the comparison with baseline value (time=-30 min) by paired t-test.

† p value for the comparison between the two visits of the study by paired t-test.

Table 1

Glucose and insulin values during the study visits.

	Time (min)	**Control Visit (water consumption)**	p*	**Leucine Visit (leucine consumption)**	p*
Glucose (mg/dl)	-30	90.0 ± 7.9		89 ± 12.3	
	0	glucose infusion			
	10	340.9 ± 40.0	< 0.001	339.6 ± 50.2	<0.001
	20	245.1 ± 25.9	<0.001	237 ± 62.0	<0.001
	30	187.5 ± 42.2	<0.001	182.6 ± 63.2	0.001
	40	144.2 ± 49.1	0.005	140.4 ± 58.5	0.013
	50	117.4 ± 42.6	0.059	105.9 ± 38.7	0.154
	60	100.7 ± 31.1	0.290	85.1 ± 31.4	0.664

Insulin (µu/ml)	-30	11.5 ± 1.7		13.6 ± 7.6	
	0	glucose infusion			
	10	55.2 ± 25.7	0.005	142.9 ± 92.7	0.005
	20	60.4 ± 38.1	0.005	166 ± 79.1	0.005
	30	42.8 ± 19.7	0.005	122.8 ± 49.4	0.005

	40	37.0 ± 19.0	0.005	88.5 ± 35.0	0.005
	50	27.3 ± 12.5	0.005	60 ± 24.5	0.005
	60	21.3 ± 9.1	0.013	42.2 ± 19.0	0.005

* p values for comparisons with baseline values (time= -30 min) by paired t-test.

6. DISCUSSION

In the present cross-over study, we showed that acute hyperglycaemia reduces FMD in healthy male volunteers, while consumption of leucine abolishes the deleterious effects of hyperglycemia on macrovascular endothelial function, probably through the observed increased insulin secretion. Indeed, although the magnitude of hyperglycaemia did not differ between the two study visits, the magnitude of hypersinsulinaemia was significantly higher in the leucine visit (leucine consumption) when compared with the control visit (water consumption). To our knowledge, this is the first study to report the favorable effect of leucine administration on postprandial endothelial dysfunction in healthy male subjects.

The detrimental effects of hyperglycaemia, both acute and chronic, on endothelial function are well established in patients with diabetes mellitus [36,56,57]. However, data about the effect of acute hyperglycemia on endothelium in healthy individuals are conflicting. Several studies have shown that acute hyperglycemia induced by an oral glucose tolerance test or intravenous infusion of glucose results in endothelial dysfunction [58-61], while others have demonstrated no significant effect on the endothelium [24,62-64]. Nevertheless, a direct comparison of these studies is precluded due to the different concentrations and routes of glucose administration and thus to the different levels and duration of hyperglycaemia and hyperinsulinaemia induced, as well as to the different methods of endothelial function evaluation.

Although the exact pathophysiology of hyperglycemia-induced endothelial dysfunction is not clearly established, several possible mechanisms have been suggested. Hyperglycaemia results in increased flux of glucose through the polyol pathway, increased intracellular production of AGEs, activation of PKC and increased activity of the hexosamine pathway [57-65]. The common unifying pathway of these pathogenic mechanisms is the increased production of ROS mediated by hyperglyceamia [24,57-65]. Moreover, hyperglycaemia is associated with a milieu of low-grade inflammation and a prothrombotic

state that both lead to endothelial cell injury [57]. More importantly, hyperglycaemia results in decreased nitric oxide bioavailability and thus impaired vasorelaxation [57].

Insulin, on the other hand, stimulates nitric oxide production in endothelial cells and has been found to be associated with increased blood flow in the microcirculation [40,41,57,66]. However, the vasodilatory effect of insulin is impaired in insulin resistant states [8,9,18], while it has been suggested that the vasodilatatory response to insulin is influenced by the level and duration of insulin exposure, the method used to evaluate blood flow and by individual factors like limb muscularity, physical fitness and the capillaries per fibre ratio [67].

Perkins et al. examined the acute effects of both hyperinsulinaemia and hyperglycaemia on endothelial function in overweight and obese participants using several clamp techniques: euinsulinaemic euglycaemic clamp, euinsulinaemic hyperglycaemic clamp, hyperinsulinaemic hyperglycaemic clamp and hyperinsulinaemic euglycaemic clamp [44]. FMD was reduced during the final 30 min of the 4-hour euinsulinaemic hyperglycaemic clamp, whereas this reduction was not observed during the hyperinsulinaemic hyperglycaemic clamp and hyperinsulinaemic euglycaemic clamp experiments. The authors concluded that hyperinsulinaemia may restore hyperglycaemia-mediated endothelial dysfunction; the same could also be the case in our study as well. Hermann et al also reported a sustained increase in endothelium-dependent vasodilation assessed by plethysmography in the forearm in healthy individuals after 4 hours of insulin infusion [68].

On the other hand, one study reported that acute hyperinsulinaemia impairs conduit vessel endothelial function, as assessed by FMD, independent of insulin sensitivity and lipid profile [43]. However, the authors of this study performed a hyperinsulinaemic euglycaemic clamp in healthy individuals and mean steady-state insulin levels were maintained 12-fold higher than the mean basal fasting insulin levels for at least 60 min. In our experiment insulin secretion was increased due to acutely induced hyperglycemia, while the peak serum insulin levels, which occurred after leucine consumption were about 10-fold higher than the baseline values and were observed 20 min after glucose infusion. Then they gradually declined until the 60 min period of our experiment. Similar results with Campia et al. [43] were reported by

another study that investigated the effect of a euglycaemic low-dose insulin clamp (insulin ~ 15.8 μu/ml) and a euglycaemic high-dose insulin clamp (insulin ~ 63.4 μu/ml) on FMD [10]. Prolonged hyperinsulineamia was found to reduce brachial endothelium-dependent vasodilation and more specifically low-dose insulin was found to reduce FMD in the 4th and 6th hour of the insulin clamp when compared with saline infusion. The contrasting results could be due to the different methods used to induce hyperinsulinaemia and to assess vasodilatation and are in no way comparable to the present study, in which participants were subjected to a more "physiological" stimulus for insulin secretion. Moreover, it should be emphasized that the duration of hyperinsulinaemia in our study was shorter (60 min).

Leucine is a branched chain amino acid with numerous metabolic roles [45,50]. It is a substrate for proteinosynthesis, it enhances insulin secretion from the β-cells and it is also involved in gluconeogenesis [45,50]. Insulin secretion from the pancreatic β-cells is increased by leucine since it serves both as a metabolic fuel and as an allosteric activator of the key enzyme glutamate dehydrogenase that acts as the β-cell leucine sensor [50].

Several studies have shown that leucine or amino acid mixtures containing leucine strongly stimulates additional insulin secretion in individuals with and without diabetes when co-ingested with carbohydrates [47-49]. In our study as well, oral leucine consumption 30 min before iv glucose infusion resulted, for the same degree of hyperglycaemia, in a higher insulin response when compared with iv glucose infusion alone in healthy male volunteers. Interestingly, although insulin AUC was significantly higher after leucine administration when compared with the control visit, glucose AUC did not differ between the two visits of our study (Figure 2). Similar findings have been reported by van Loon et al, who described that ingestion of carbohydrate only drink vs. carbohydrate plus amino acid mixture drink did not result in significantly different plasma glucose concentrations within the 2-hours time frame of the experiment in 10 healthy male participants [48]. This was partly attributed to the increased glucagon plasma concentrations observed after mixture drink ingestion and to the short time frame of the experiment. Although we did not measure glucagon concentration, this could also apply for our study too, while glucose levels did indeed differ significantly between the two

visits of our study only in 60 min measurement. Moreover, we showed that the negative effect of acute hyperglycaemia on endothelial function was abolished by the oral pre-administration of leucine. Therefore, it seems reasonable to conclude that leucine ameliorates the hyperglycemia-induced reduction of FMD through an increased insulinaemic response. This finding further supports the vasodilatory effect of acute hyperinsulinaemia reported in other studies as well [44,68].

A main limitation of our study is the small number of participants as well as the short duration of the experiment. However, strengths of our study are the rather “physiological” stimulus employed for insulin secretion and the controlled conditions under which the experiment was performed.

To summarize, the present study showed that acute hyperglycaemia has a detrimental effect on endothelial function in healthy male individuals, while oral leucine administration before iv glucose infusion results in higher insulin concentrations when compared with iv glucose infusion alone. Moreover, we showed that for the same degree of hyperglycaemia, a further increase in serum insulin levels induced by the consumption of leucine prevents hyperglycaemia-mediated endothelial dysfunction. These findings suggest that acute hyperinsulinaemia, even in the face of high glucose serum levels, has a favorable effect on the endothelium. Since endothelial dysfunction is one of the earliest pathophysiological precursors to atherosclerotic cardiovascular disease and is implicated in both initiation and progression of atherosclerosis, future research is needed to investigate the mechanisms and the possible ways to preserve endothelial function in the postprandial state in both healthy individuals and more importantly in patients with diabetes.

7. CONCLUSIONS

Acute hyperglycaemia impairs endothelial function in healthy male individuals. Oral leucine administration prevents hyperglycaemia-mediated endothelial dysfunction probably due to enhanced insulin secretion. Further studies are needed to examine the mechanisms and the possible ways to preserve endothelial function in the postprandial state.

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