

Systemic inhibition of nitric oxide synthesis in non-diabetic individuals produces a significant deterioration in glucose tolerance by increasing insulin clearance and inhibiting insulin secretion

A. Natali · R. Ribeiro · S. Baldi · A. Tulipani · M. Rossi · E. Venturi · A. Mari · M. P. Macedo · E. Ferrannini

Received: 9 October 2012 / Accepted: 31 December 2012 / Published online: 31 January 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract

Aims/hypothesis Endogenous NO inhibits insulin release in isolated beta cells and insulin-degrading enzyme activity in hepatocytes, while NO release from endothelial cells has been suggested to enhance insulin action. We assessed the overall effect of systemic inhibition of endogenous NO synthesis on glucose homeostasis in humans.

Methods Twenty-four non-diabetic volunteers underwent two hyperglycaemic (+7 mmol/l) clamps with either saline or L-NG-nitroarginine methyl ester (L-NAME, at rates of 2.5, 5, 10 and 20 $\mu\text{gmin}^{-1}\text{kg}^{-1}$) infusion. Another five volunteers underwent an OGTT with either saline or L-NAME (20 $\mu\text{gmin}^{-1}\text{kg}^{-1}$) infusion. Blood pressure and heart rate were measured to monitor NO blockade; during the OGTT, endothelial function was assessed by peripheral arterial tonometry and insulin secretion by C-peptide deconvolution and insulin secretion modelling.

Results Compared with saline, L-NAME at the highest dose raised mean blood pressure (+20±2 mmHg), depressed heart rate (-12±2 bpm) and increased insulin clearance (+50%).

Electronic supplementary material The online version of this article (doi:10.1007/s00125-013-2836-x) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

A. Natali (✉) · S. Baldi · A. Tulipani · M. Rossi · E. Venturi · E. Ferrannini
Dipartimento di Medicina Clinica e Sperimentale,
Università di Pisa, Via Roma 67,
56100 Pisa, Italy
e-mail: andrea.natali@med.unipi.it

A. Natali · R. Ribeiro · M. P. Macedo
Centro de Estudos de Doenças Crónicas, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisbon, Portugal

A. Mari
Institute of Biomedical Engineering, National Research Council, Padova, Italy

First-phase insulin secretion was impaired, but insulin sensitivity (*M/I* index) was unchanged. During the OGTT, L-NAME raised 2 h plasma glucose by 1.8 mmol/l ($p<0.01$), doubled insulin clearance and impaired beta cell glucose sensitivity while depressing endothelial function.

Conclusions/interpretation In humans, systemic NO blockade titrated to increase blood pressure and induce endothelial dysfunction does not affect insulin action but significantly impairs glucose tolerance by increasing plasma insulin clearance and depressing insulin secretion, namely first-phase and beta cell glucose sensitivity.

Keywords Insulin clearance · Insulin secretion · Insulin sensitivity · Nitric oxide

Abbreviations

GIR	Glucose infusion rates
IDE	Insulin-degrading enzyme
ISR	Insulin secretion rate
L-NAME	L-NG-nitroarginine methyl ester
NOS	Nitric oxide synthase

Introduction

Both insulin and glucose promote NO synthesis in tissues (endothelium, beta cells, skeletal muscle, liver and brain) that participate in the maintenance of glucose homeostasis [1, 2]. The reduced NO bioavailability that is often found in conditions of impaired glucose homeostasis is expected to involve most of these tissues since it is sustained by enhanced oxidative stress, a systemic process.

Despite a large number of experimental studies and a few clinical studies, whether and to what extent systemic NO production participates in the physiological regulation of glucose metabolism in humans remains undetermined. Studies utilising competitive inhibitors (arginine analogues) to block constitutive NO synthesis have demonstrated effects on glucose control that are rather heterogeneous both within and among different tissues. In isolated beta cells, the physiological glucose-induced rise in NO is permissive for insulin release, but higher NO concentrations dose-dependently curtail glucose-stimulated insulin secretion [3–5]. In intact skeletal muscle tissue (perfused limb protocols in animal models and humans), insulin promotes endothelial NO release, thereby allowing optimal tissue perfusion and deployment of a full metabolic action [6]. Exposure of isolated skeletal muscle cells to NO promotes glucose transport via an insulin-independent pathway [7].

In the liver, studies in animals have found that physiological endogenous NO production has a minor effect on hepatic glucose production by increasing both glycogenolysis [8] and glucose uptake [9]. In humans, the infusion of the arginine analogue L-NG-nitroarginine methyl ester (L-NAME) raised blood pressure but had no effect on fasting hepatic glucose production [10]. Other studies showing a direct role of NO in inducing hepatic insulin resistance have used models in which excess NO was generated through liver-specific overexpression of the inducible nitric oxide synthase (NOS) isoform [11]. In liver homogenates, NO has recently been shown to dose-dependently inhibit the specific protease insulin-degrading enzyme (IDE), which is largely responsible for whole-body insulin clearance [12]. The physiological relevance of this effect has not been assessed either in experimental animals or in humans. Finally, in anaesthetised rats, blocking NO synthesis selectively in the central nervous system with an intracerebroventricular L-NAME infusion produced a mild impairment of both peripheral insulin sensitivity and arginine-induced insulin secretion [13].

On aggregate, the available information indicates that NOS inhibition in the beta cell would result in an enhanced insulin secretion, while in the liver would produce an upregulation of insulin degradation and in the peripheral tissue (acting on tissue perfusion and on the central nervous system) would induce insulin resistance. The present study was undertaken to measure the size of these effects during a partial and systemic NO synthesis inhibition in humans, and to verify whether these multiple changes eventually lead to deterioration of glucose tolerance.

Methods

Study design We first evaluated the effect of increasing L-NAME infusion rates (2.5, 5, 10 and 20 $\mu\text{g}^{-1}\text{min}^{-1}\text{kg}^{-1}$)

on blood pressure, heart rate and insulin secretion, action and clearance using a 2-h hyperglycaemic clamp. Next we evaluated the effect of the highest L-NAME dose (20 $\mu\text{g}^{-1}\text{min}^{-1}\text{kg}^{-1}$) on glucose tolerance, insulin secretion and insulin clearance during a standard OGTT, with simultaneous measurements of blood pressure and endothelial function.

Participants A total of 29 non-diabetic volunteers of either sex (ten women and 19 men, age 39 ± 7 years, BMI 27 ± 4 kg/m^2) were recruited from hospital employees and students and from the outpatient clinic. Each participant received a standard OGTT to exclude diabetes and signed a written informed consent on which the study details and its potential risks were described. This investigation was approved by the institutional review board and was carried out in accordance with the Declaration of Helsinki as revised in 2000 (www.vma.net/e/policy/b3.htm).

Four groups of five participants each underwent two consecutive hyperglycaemic clamp studies 1–2 weeks apart, performed in single-blind fashion and random order with respect to L-NAME (2.5, 5, 10 or 20 $\mu\text{g}^{-1}\text{min}^{-1}\text{kg}^{-1}$) or vehicle (normal saline) infusion. Four participants received saline twice (L-NAME 0) to establish reproducibility. The remaining five individuals underwent two consecutive OGTTs during an L-NAME (20 $\mu\text{g}^{-1}\text{min}^{-1}\text{kg}^{-1}$) or saline infusion administered in single-blind fashion and in random order.

Hyperglycaemic clamp Participants arrived at the metabolic ward at 8:00 hours after an overnight fast. A 20 G polyethylene catheter was placed in a retrograde manner into a wrist vein, and the hand was placed in a heating pad for sampling of arterialised blood. A second cannula was inserted into an antecubital vein for insulin, glucose and test drug infusion. After baseline blood sampling at time -30 min, either saline or L-NAME was infused throughout the study until time 130 min. After drawing two further blood samples (-10 and 0 min), a glucose bolus, calculated to rapidly increase the glucose concentration into the readily accessible glucose distribution volume (150 ml/kg), was delivered in 90 s, followed by a continuous glucose infusion adjusted to achieve and maintain plasma glucose levels of 7.0 mmol/l above fasting values for 120 min. Plasma glucose was measured (Glucose Analyser; Beckman, Fullerton, CA, USA) every 5–10 min, and blood samples for hormone measurements were collected every 2 min for the first 10 min and every 20 min until 120 min. At 120 min, an arginine bolus (5 g in a 20% water solution) was injected, and blood was collected every 2 min for the next 10 min. Heart rate and blood pressure were measured using an automatic device (Press-Mate; Colin, Courbevoie, France).

OGTT Participants arrived at the metabolic ward at 08:00 hours after an overnight fast. A cannula was inserted

into an antecubital vein in the left arm for blood-drawing. After two baseline samples at time -30 and 0 min, participants drank 75 g of a glucose solution (50% in water) and blood sampling was repeated at time 15 , 30 , 60 , 90 and 120 min. Either saline or L-NAME ($20 \mu\text{g}^{-1}\text{min}^{-1}\text{kg}^{-1}$) infusion was started at time -30 min, and continued to 120 min. At -30 , 0 , 60 and 120 min, heart rate, blood pressure and basal and postischaemic peripheral arterial tonometry (Endo-PAT2000; ItamarMedical, Caesarea, Israel) measurements were taken to evaluate the haemodynamic effects of the L-NAME infusion.

Analytical determinations Plasma insulin and C-peptide were measured using an electrochemiluminescence immune assay method (Cobas E411; Roche, Rotkreuz, Switzerland).

Data analysis Glucose infusion rates (GIRs) during the clamp were averaged over 10-min intervals, and the mean value between times 60 and 120 min was considered the steady-state GIR; glucose utilisation (M value) was then calculated by correcting the 60 – 120 -min GIR for the change in the body glucose pool (assuming a glucose distribution volume of 200 ml/kg body weight). Individual insulin sensitivity (M/I) was expressed as the M value normalised for the mean 60 – 120 -min plasma insulin concentration. Insulin secretion rate (ISR), expressed in pmolmin^{-1} per m^2 of body surface area, was calculated by deconvolution of plasma C-peptide data according to the method of van Cauter et al [14]. First-phase insulin secretion was defined as the area under the ISR curve (ISR-AUC) between time 0 and 8 min, second-phase insulin secretion as the ISR-AUC from 60 to 120 min, and the arginine response as the ISR-AUC from 120 to 130 min. AUCs were calculated by the trapezoid rule. Insulin clearance, expressed in l/min per m^2 of body surface area, was estimated from the ratio of ISR and insulin AUC. During the OGTT, ISR was calculated by deconvolution of plasma C-peptide concentrations. Insulin clearance during the OGTT was estimated as the ratio of ISR-AUC to the corresponding insulin AUC.

Beta cell function was assessed using a model that describes the relationship between insulin secretion and glucose concentration, which has previously been illustrated in detail [15, 16]. Briefly, the model expresses insulin secretion as the sum of two components. The first component represents the dependence of insulin secretion on absolute glucose concentration through a dose–response function; the second component, termed *rate sensitivity*, represents the dependence of insulin secretion on the rate of change of glucose concentration. Characteristic variables of the dose–response are the slope within the observed glucose range, named the beta cell *glucose sensitivity*, and the insulin secretion at a fixed glucose concentration value (usually 5 mmol/l), denoted as *fasting secretory tone*. The dose–

response is also modulated by a potentiation factor, which is a function of time and is named the *potentiation ratio*.

Statistical analysis Paired differences were tested by the Wilcoxon sign-rank test, and differences between plasma concentration curves with the use of ANOVA for repeated measures. A p value ≤ 0.05 was considered statistically significant.

Results

Hyperglycaemic clamp In response to a square wave of hyperglycaemia ($+7$ mmol/l), ISR and plasma insulin concentrations displayed the typical biphasic pattern (Fig. 1); in both the L-NAME 0 (saline) and L-NAME 2.5 experiments, the responses were superimposable for the two occasions (electronic supplementary material [ESM] Fig. 1). As L-NAME infusion was escalated, we observed progressively lower plasma insulin concentrations but superimposable ISR (Fig. 1) and plasma C-peptide (data not shown). With both L-NAME 10 and 20 , the plasma insulin curve was statistically different from that for saline (ANOVA for repeated measures, $p=0.04$ and $p=0.001$, respectively). On average, with L-NAME 20 plasma insulin concentrations were 50% lower than with saline, resulting in significantly lower GIRs ($p=0.001$) (Fig. 1). The analysis of aggregated values (Table 1) shows that none of the L-NAME doses affected fasting variables. L-NAME 2.5 , 5 and 10 had no effect on insulin secretion, clearance or action, while L-NAME 20 depressed first-phase insulin secretion, doubled insulin clearance and lowered the M value. However, when M values were corrected for the prevailing insulin concentration (M/I) there was no difference between L-NAME 20 and saline.

In all the saline experiments, arterial blood pressure and heart rate were stable throughout all the study phases (data not shown); they were stable also with L-NAME 2.5 and 5 , whereas blood pressure increased and heart rate decreased in proportion to infusion rates in the L-NAME 10 and 20 studies (Fig. 2). Interestingly, the administration of arginine was associated with a prompt decline in blood pressure and a rise in heart rate in the L-NAME 5 , 10 and 20 experiments.

OGTT L-NAME infusion at $20 \mu\text{gmin}^{-1}\text{kg}^{-1}$ induced a significant deterioration in glucose tolerance, which was more prominent during the second hour of the test (Fig. 3; $p<0.01$ by ANOVA for repeated measures). Correspondingly, insulin secretion was delayed and blunted, as evident from the plasma C-peptide and insulin levels and the ISRs (Fig. 3b–d). To assess the adequacy of the insulin secretory response to the glucose values achieved during the OGTT, we calculated beta cell glucose sensitivity (i.e. the slope of the

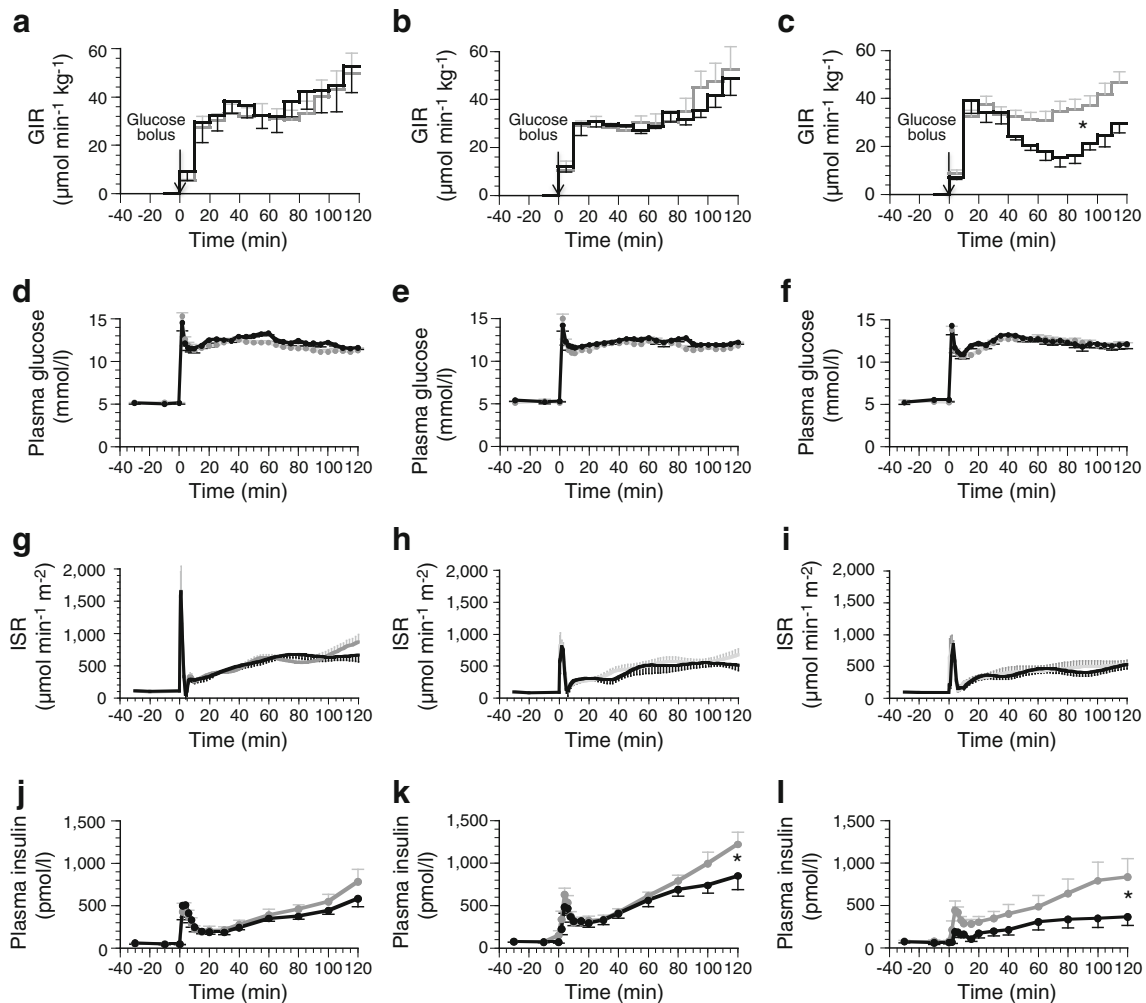


Fig. 1 Time course of exogenous GIR (a–c), plasma glucose (d–f), ISR (g–i) and plasma insulin (j–l) during the hyperglycaemic clamp tests carried out with a concomitant i.v. L-NAME 5 (a, d, g, j), 10 (b, e,

h, k) and 20 (c, f, i, l) $\mu\text{gmin}^{-1}\text{kg}^{-1}$ (black lines) or saline (grey lines) infusion. Small vertical lines represent the SEM values. * $p < 0.05$ with respect to saline by ANOVA for doubly repeated measures

relationship between ISR and plasma glucose). As shown in Fig. 3e, L-NAME caused a 40% decrease in beta cell glucose sensitivity (from 121 ± 15 to 75 ± 6 $\text{pmolmin}^{-1}\text{m}^{-2}$ per mmol/l ; $p = 0.031$). Interestingly, total insulin secretion (40 ± 3 vs 37 ± 2 nmol/m^2) and the other model-derived variables (potentiation 0.99 ± 0.08 vs 1.58 ± 0.32 ; rate sensitivity $1,117 \pm 154$ vs 835 ± 237 pmolm^{-2} per mmol/l) were similar, while the fasting secretory tone (from 90 ± 16 to 108 ± 13 $\text{pmolmin}^{-1}\text{m}^{-2}$ at 5.0 mmol/l glucose; $p < 0.02$) was upregulated by L-NAME.

Plasma insulin removal during the OGTT, indirectly estimated from the slope of the scatterplot of plasma insulin concentration and the corresponding ISR, was 50% higher with L-NAME 20 than saline ($p = 0.04$ by slope comparison test) (Fig. 3f). Interestingly, L-NAME had no effect on plasma insulin clearance in the fasting state (saline 1.69 ± 0.18 vs L-NAME 1.68 ± 0.15 $\text{l min}^{-1}\text{m}^{-2}$), but completely prevented the physiological decline in insulin clearance observed during the OGTT (saline 1.07 ± 0.16 vs L-NAME 1.72 ± 0.29 $\text{l min}^{-1}\text{m}^{-2}$; $p < 0.01$).

A progressive increase in mean blood pressure and a progressive decline in endothelial function were recorded during the 2 h of the OGTT (Fig. 4).

Discussion

This study demonstrates that systemic NOS blockade—in a degree that increases blood pressure—causes a clinically significant deterioration of glucose tolerance, which results from the combination of beta cell dysfunction and enhanced degradation of secreted insulin, while peripheral insulin sensitivity is unaffected. The current data thus demonstrate a novel mechanism by which glucose homeostasis is maintained and is linked to vascular health. Following nutrient ingestion, NO production is physiologically enhanced in the liver, by activation of the local parasympathetic cholinergic discharge [17], and systemically, by the concomitant rise in

Table 1 Aggregated values of insulin secretion, insulin clearance and insulin sensitivity

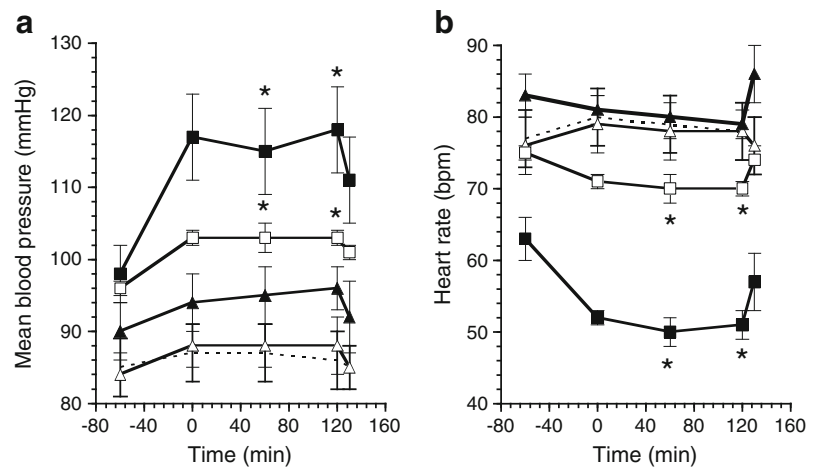
Variable	L-NAME 0		L-NAME 2.5		L-NAME 5		L-NAME 10		L-NAME 20	
	Saline	Saline	Saline	L-NAME	Saline	L-NAME	Saline	L-NAME	Saline	L-NAME
ISR basal (pmol min ⁻¹ m ⁻²)	68±10	85±22	69±17	77±10	94±9	94±9	86±13	86±12	92±9	87±10
Insulin cl. basal (l min ⁻¹ m ⁻²)	0.915±0.119	1.136±0.110	1.305±0.139	1.597±0.178	2.242±0.300	2.097±0.353	1.217±0.223	1.223±0.144	1.281±0.179	1.492±0.211
ISR first-phase (pmol min ⁻¹ m ⁻²)	489±92	472±89	564±128	501±88	600±92	666±106	456±20	397±31	428±65	343*±40
Insulin cl. first-phase (l min ⁻¹ m ⁻²)	1.378±0.048	1.519±0.062	1.895±0.194	1.659±0.142	2.048±0.274	2.129±0.319	1.178±0.163	1.227±0.138	1.385±0.071	2.552*±0.246
ISR 60–120 (pmol min ⁻¹ m ⁻²)	527±89	505±85	492±111	479±64	650±66	647±37	568±67	526±63	480±87	445±55
Insulin cl. 60–120 (l min ⁻¹ m ⁻²)	1.039±0.085	1.059±0.149	1.574±0.048	1.895±0.232	1.335±0.182	1.579±0.180	0.650±0.081	0.772±0.114	0.833±0.164	2.039*±0.528
ISR arginine (pmol min ⁻¹ m ⁻²)	1,134±180	1,208±228	1,558±189	1,504±227	2,114±134	2,350±238	1,733±211	1,475±209	1,392±115	1,415±107
Insulin cl. arginine (l min ⁻¹ m ⁻²)	0.564±0.161	0.605±0.045	1.111±0.188	1.038±0.163	0.683±0.072	0.857±0.108	0.465±0.048	0.433±0.035	0.568±0.094	1.106*±178
M (μmol min ⁻¹ kg ⁻¹)	37.5±4.9	39.9±3.2	41.3±4.8	40.0±3.4	41.5±6.9	46.1±8.5	41.4±6.8	38.2±3.1	39.3±4.8	20.4*±2.7
M/I (μmol min ⁻¹ kg ⁻¹ [nmol/l] ⁻¹)	64±21	76±15	125±24	150±33	58±8	72±13	36±7	40±7	83±36	104±39

First-phase=time 0–10 min; 60–120=mean value from min 60 to 120 into the clamp; Arginine=10 min after arginine injection

**p*<0.05, for the comparison L-NAME vs saline by Wilcoxon test

cl., clearance

Fig. 2 Mean blood pressure (a) and heart rate (b) measured during the hyperglycaemic clamp tests carried out with a concomitant i.v. infusion of L-NAME 0 (dotted line), 2.5 (white triangles), 5 (black triangles), 10 (white squares) and 20 (black squares) $\mu\text{gmin}^{-1}\text{kg}^{-1}$. At 120 min, an i.v. bolus of arginine (4 g) was administered. $*p<0.05$ with respect to baseline (-60 min) values

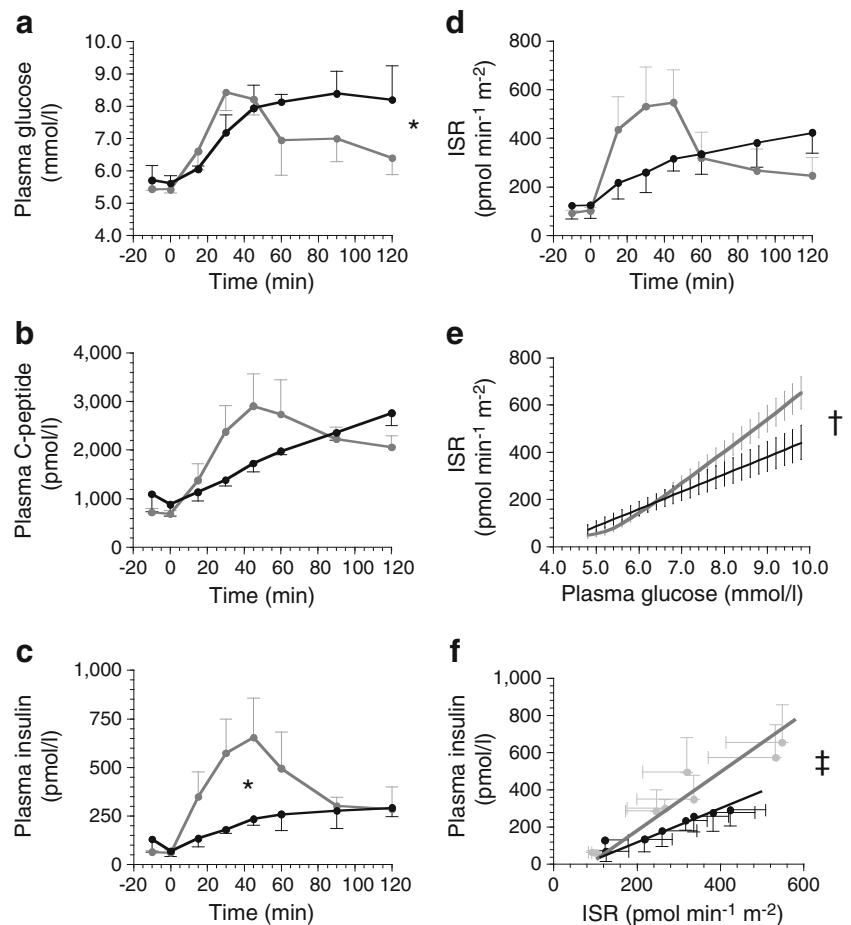


plasma insulin levels (known to activate endothelial NOS). By inhibiting plasma insulin clearance, the NO response reduces the burden on the beta cells. Conceivably, when NOS activity is depressed, posthepatic insulin availability is reduced, and beta cells are required to release more insulin to achieve the same plasma insulin concentration. In addition, reduced NO bioavailability directly depresses beta cell glucose sensitivity, thereby further challenging postprandial glycaemic control. Our data explain the findings of a recent

study in healthy participants [18], which, in addition to demonstrating that L-NAME does not interfere with gastric emptying during an OGTT (the main objective of the study), also reported higher glucose excursions coupled with lower insulin levels.

One possible mechanism by which L-NAME increases insulin clearance could be by blood flow redistribution. Insulin degradation in liver and muscle is dependent on blood flow (whereas in the kidney it is not, given the high

Fig. 3 Results of the OGTT tests carried out with either saline (grey lines) or L-NAME 20 $\mu\text{gmin}^{-1}\text{kg}^{-1}$ (black lines). Time course of plasma glucose (a), C-peptide (b) and insulin (c) concentrations. (d) Time course of ISR. (e) Insulin secretion vs glucose dose–response curves. (f) Scatterplot of plasma insulin concentration and ISR, averaged on a 10-min interval around the blood sampling time). $*p<0.05$ for differences between curves by ANOVA for repeated measures; $^{\ddagger}p<0.05$ for differences between curves by Wilcoxon sign-rank test; $^{\ddagger}p<0.05$ by slope comparison test



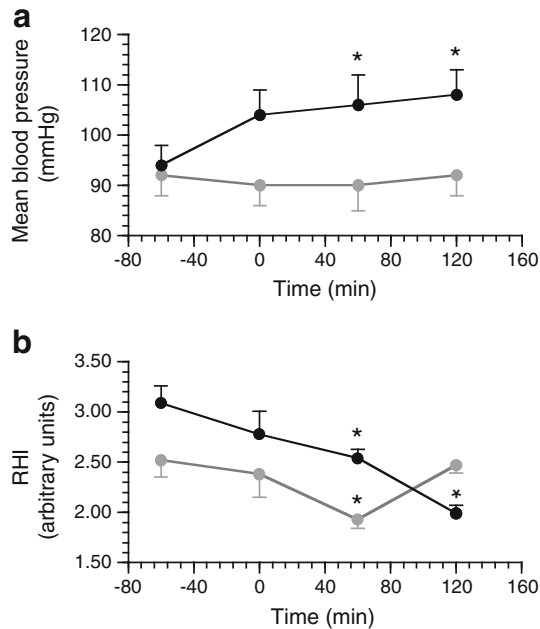


Fig. 4 Mean blood pressure (**a**) and endothelial function (**b**) as measured by peripheral arterial tonometry at baseline and after 5 min of ischaemia (reactive hyperaemia index [RHI]) during the OGTT tests carried out with a concomitant L-NAME $20 \mu\text{gmin}^{-1}\text{kg}^{-1}$ (black line) or saline (grey line) i.v. infusion. * $p < 0.05$ with respect to time by ANOVA for repeated measures

perfusion rate of this organ). We did not measure tissue blood flow; however, data from experimental studies do not support this possibility. In rats, intraportal L-NAME administration reduces liver perfusion [19], while systemic L-NAME has no effect on portal haemodynamics despite a 60% increase in arterial blood pressure [20]. In skeletal muscle, systemic L-NAME infusion either does not affect blood flow to muscle tissues or reduces it (as elegantly demonstrated by Majumdar et al [21]). The acute increase in blood pressure, with the attendant hormonal response, is also unlikely to be involved: a similar rise in blood pressure, achieved with angiotensin II infusion, had no effect on insulin clearance in humans [22]. By inducing a reflex depression of the sympathetic nervous system [23], L-NAME should, if anything, reduce insulin clearance (which is activated by adrenergic agonists [22]). We therefore suggest that the effect of L-NAME on insulin clearance is a direct one and is likely to involve liver IDE, the chief enzyme system for insulin degradation, which has been shown in vitro to be inhibited by NO: the lower the NO availability, the stronger the activation of the enzyme [12].

Clearly, our data describe an acute phenomenon and cannot predict whether chronic NOS inhibition would result in a persistent decline in glucose tolerance. In rats, the administration of L-NAME in drinking water for 14 days resulted in a deterioration of glucose tolerance coupled with an early insulin secretion defect [24]. Under more extreme

conditions, liver-selective IDE knockout mice show an initial improvement in glucose tolerance, but with ageing the animals develop insulin resistance secondary to chronic hyperinsulinisation and insulin receptor downregulation [25]. In clinical conditions characterised by chronic, low-grade inflammation, liver inducible NOS activation might reduce insulin clearance. In fact, obese individuals show a degree of hyperinsulinaemia that is neither entirely explained by the degree of obesity and/or insulin resistance nor fully accounted for by saturation of insulin removal [26].

With regard to the haemodynamic effects of NOS blockade, we documented a dose-dependent rise in arterial blood pressure and fall in heart rate (Fig. 2) as well as a recovery of both variables upon adding arginine, the precursor of NO. These effects, and the significant decline in endothelial function (Fig. 4), mimic the pathological features of essential hypertension, a model of chronically reduced vascular NO availability. However, it should be noted that essential hypertension has been associated with reduced insulin clearance [27]. This discrepancy is likely to be explained by the fact that essential hypertension is frequently associated with chronic primary insulin resistance and hyperinsulinaemia, which, in turn, is associated with reduced insulin clearance (due to saturation of first-pass insulin removal). The effect on insulin clearance we report here is independent of insulin secretion.

During both intravenous and oral glucose stimulation, an inhibitory effect of L-NAME on beta cell function was also observed, manifesting itself as reduced first-phase insulin release and beta cell glucose sensitivity, respectively. This finding may suggest the involvement of gastrointestinal hormones. In this regard, however, Gentilcore et al [18] reported that neither the glucagon-like peptide 1 nor the gastrointestinal peptide (GIP) response to an OGTT was modified by L-NAME (infused at rates inducing blood pressure and heart rate changes very similar to ours). Therefore, neural control mechanisms, such as the parasympathetic cholinergic outflow to the pancreas (the cephalic phase of insulin secretion [28, 29]), may be involved. It is also pertinent to note that the inhibitory effect of L-NAME on insulin secretion is in contrast with previous work. We and others [3–5] have reported that, in rodent beta cell cultures, endogenous NO production does inhibit insulin secretion. This discrepancy could be due to differences in species or experimental conditions.

The lack of effect on peripheral insulin sensitivity conflicts with data in humans showing that L-NAME infusion reduces insulin-dependent glucose metabolism by preventing optimal tissue perfusion. However, the discrepancy is only apparent since this effect is in quantitative terms either negligible or extremely small. In fact, the first study showing that the forearm vasodilatation occurring during systemic insulin infusion could be prevented with L-NAME reported no concomitant reduction in whole-body glucose uptake [30]. Similarly, the study that demonstrated that

L-NAME could abolish insulin-induced vasodilatation in one leg was not associated with a significant effect on whole-body glucose disposal since the non-vasodilated leg showed an almost fully compensatory increase in fractional glucose extraction [31]. On the other hand, supplying NO to the forearm of individuals with insulin resistance did not produce an improvement in the limb's insulin-stimulated glucose uptake [32, 33]. Finally, our study design is not adequate to detect minor changes (less than 25%) in insulin sensitivity; the hyperglycaemic clamp test is not a tool as sensitive as the euglycaemic clamp in measuring insulin sensitivity (essentially because the insulin–glucose dose–response curve is not linear) and the size of each study group is small. However, it is important to note that the trend for M/I was to increase with L-NAME.

One possible limitation of this study is that the study groups were rather heterogeneous with respect to insulin sensitivity, insulin clearance and blood pressure as shown in Table 1 and Fig. 3. However, given the paired study design, the high reproducibility of the tests (saline vs saline and saline vs L-NAME 2.5; see ESM Fig. 1) and the size of the effects, we consider it unlikely that the differences between the groups might have affected our main results.

In summary, we have shown that systemic NOS blockade raises blood pressure and generates endothelial dysfunction at the same time as inducing a clinically significant deterioration of glucose tolerance, due to increased insulin clearance and impaired insulin secretion.

Acknowledgements We wish to thank S. Burchielli for her technical assistance.

Source of funding The study received partial support from FTC (Fundação para a Ciência e a Tecnologia - Portuguese Foundation for Science and Technology) under the project PTDC/DTP-EPI/0207/2012. Other funds came from resources made available by other funded studies.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement All authors substantially contributed to the conception and design of the study, acquisition or analysis of data and interpretation of the data or drafting of the manuscript. The article has been critically revised and has received final approval for publication by all authors.

References

- Barrett EJ, Eggleston EM, Inyard AC et al (2009) The vascular actions of insulin control its delivery to muscle and regulate the rate-limiting step in skeletal muscle insulin action. *Diabetologia* 52:752–764
- Huang PL (2009) eNOS, metabolic syndrome and cardiovascular disease. *Trends Endocrinol Metab* 20:295–302
- Lajoix AD, Pugniere M, Roquet F et al (2004) Changes in the dimeric state of neuronal nitric oxide synthase affect the kinetics of secretagogue-induced insulin response. *Diabetes* 53:1467–1474
- Natali A, Santini E, Delbarba A et al (2012) Effects of short and prolonged mild intracellular nitric oxide manipulations on various aspects of insulin secretion in INS-1E beta-cells. *Exp Clin Endocrinol Diabetes* 120:210–216
- Beffy P, Lajoix AD, Masiello P et al (2001) A constitutive nitric oxide synthase modulates insulin secretion in the INS-1 cell line. *Mol Cell Endocrinol* 183:41–48
- Barrett EJ, Wang H, Upchurch CT, Liu Z (2011) Insulin regulates its own delivery to skeletal muscle by feed-forward actions on the vasculature. *Am J Physiol Endocrinol Metab* 301:E252–E263
- Deshmukh AS, Long YC, de Castro BT et al (2010) Nitric oxide increases cyclic GMP levels, AMP-activated protein kinase (AMPK) α 1-specific activity and glucose transport in human skeletal muscle. *Diabetologia* 53:1142–1150
- Borgs M, Bollen M, Keppens S, Yap SH, Stalmans W, Vanstapel F (1996) Modulation of basal hepatic glycogenolysis by nitric oxide. *Hepatology* 23:1564–1571
- An Z, Winnick JJ, Farmer B et al (2010) A soluble guanylate cyclase-dependent mechanism is involved in the regulation of net hepatic glucose uptake by nitric oxide in vivo. *Diabetes* 59:2999–3007
- Sprangers F, Jellema WT, Lopuhaa CE et al (2002) Partial inhibition of nitric oxide synthesis in vivo does not inhibit glucose production in man. *Metab Clin Exp* 51:57–64
- Fujimoto M, Shimizu N, Kunii K, Martyn JA, Ueki K, Kaneki M (2005) A role for iNOS in fasting hyperglycemia and impaired insulin signaling in the liver of obese diabetic mice. *Diabetes* 54:1340–1348
- Cordes CM, Bennett RG, Siford GL, Hamel FG (2011) Redox regulation of insulin degradation by insulin-degrading enzyme. *PLoS One* 6:e18138
- Shankar RR, Wu Y, Shen HQ, Zhu JS, Baron AD (2000) Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance. *Diabetes* 49:684–687
- Van Cauter E, Mestrez F, Sturis J, Polonsky KS (1992) Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes* 41:368–377
- Mari A, Schmitz O, Gastaldelli A, Oestergaard T, Nyholm B, Ferrannini E (2002) Meal and oral glucose tests for the assessment of β -cell function: modeling analysis in normal subjects. *Am J Physiol Endocrinol Metab* 283:E1159–E1166
- Mari A, Tura A, Gastaldelli A, Ferrannini E (2002) Assessing insulin secretion by modeling in multiple-meal tests: role of potentiation. *Diabetes* 51(Suppl 1):S221–S226
- Lautt WW, Macedo MP, Sadri P, Takayama S, Duarte Ramos F, Legare DJ (2001) Hepatic parasympathetic (HISS) control of insulin sensitivity determined by feeding and fasting. *Am J Physiol Gastrointest Liver Physiol* 281:G29–G36
- Gentilcore D, Visvanathan R, Russo A et al (2005) Role of nitric oxide mechanisms in gastric emptying of, and the blood pressure and glycemic responses to, oral glucose in healthy older subjects. *Am J Physiol Gastrointest Liver Physiol* 288:G1227–G1232
- Moore MC, Dicostanzo CA, Smith MS et al (2008) Hepatic portal venous delivery of a nitric oxide synthase inhibitor enhances net hepatic glucose uptake. *Am J Physiol Endocrinol Metab* 294: E768–E777
- Li X, Benjamin IS, Alexander B (2003) The role of nitric oxide in systemic and hepatic haemodynamics in the rat in vivo. *N Schmied Arch Pharmacol* 368:142–149
- Majumdar S, Genders AJ, Inyard AC, Frison V, Barrett EJ (2012) Insulin entry into muscle involves a saturable process in the vascular endothelium. *Diabetologia* 55:450–456

22. O'Callaghan CJ, Komersova K, Louis WJ (1998) Acute effects of blood pressure elevation on insulin clearance in normotensive healthy subjects. *Hypertension* 31:104–109
23. Wecht JM, Weir JP, Goldstein DS et al (2008) Direct and reflexive effects of nitric oxide synthase inhibition on blood pressure. *Am J Physiol Heart Circ Physiol* 294:H190–H197
24. Balon TW, Jasman AP, Young JC (1999) Effects of chronic N (omega)-nitro-L-arginine methyl ester administration on glucose tolerance and skeletal muscle glucose transport in the rat. *Nitric Oxide* 3:312–320
25. Abdul-Hay SO, Kang D, McBride M, Li L, Zhao J, Leissring MA (2011) Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance. *PLoS One* 6: e20818
26. Goodarzi MO, Cui J, Chen YD, Hsueh WA, Guo X, Rotter JJ (2011) Fasting insulin reflects heterogeneous physiological processes: role of insulin clearance. *Am J Physiol Endocrinol Metab* 301:E402–E408
27. Lender D, Arauz-Pacheco C, Adams-Huet B, Raskin P (1997) Essential hypertension is associated with decreased insulin clearance and insulin resistance. *Hypertension* 29:111–114
28. Ahren B, Holst JJ (2001) The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and non-cholinergic mechanisms and is important for postprandial glycaemia. *Diabetes* 50:1030–1038
29. D'Alessio DA, Kieffer TJ, Taborsky GJ Jr, Havel PJ (2001) Activation of the parasympathetic nervous system is necessary for normal meal-induced insulin secretion in rhesus macaques. *J Clin Endocrinol Metab* 86:1253–1259
30. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P (1994) Nitric oxide release accounts for insulin's vascular effects in humans. *J Clin Invest* 94:2511–2515
31. Baron AD, Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G (1995) Insulin-mediated skeletal muscle vasodilation contributes to both insulin sensitivity and responsiveness in lean humans. *J Clin Invest* 96:786–792
32. Natali A, Galvan AQ, Pecori N, Sanna G, Toschi E, Ferrannini E (1998) Vasodilation with sodium nitroprusside does not improve insulin action in essential hypertension. *Hypertension* 31:632–636
33. Natali A, Sironi AM, Toschi E et al (2000) Effect of vitamin C on forearm blood flow and glucose metabolism in essential hypertension. *Arterioscler Thromb Vasc Biol* 20:2401–2406