Systemic nitric oxide synthase inhibition increases insulin sensitivity in man

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(Received 25 June/10 September 1997; accepted 22 September 1997)

INTRODUCTION

Insulin resistance is a hallmark of obesity, hypertension and non-insulin-dependent diabetes mellitus (NIDDM) and is thought to contribute to the increased cardiovascular mortality in large prospective epidemiological studies [1, 2]. If we are to develop specific therapies for insulin resistance then we have to understand more about which endogenous factors influence insulin sensitivity.

Insulin itself has a specific action to vasodilate skeletal muscle vasculature in humans [3]. This effect appears blunted in several insulin-resistant states including hypertension [4], obesity [5] and NIDDM [6]. Insulin's ability to increase blood flow means that insulin is able to increase the delivery of both itself and its major substrate, glucose, to the skeletal muscle cell. Therefore, it is logical to think that insulin-mediated vasodilatation might be a determinant of insulin sensitivity [7]. Recent observations suggest that insulin-mediated vasodilatation may be nitric oxide (NO)-dependent [8, 9] and have led to the hypothesis that insulin sensitivity itself could be determined at least in part by NO [10]. Until now only local infusion studies have been performed which have reported a decrease in limb glucose uptake when N(G)-monomethyl L-arginine (L-NMMA) has been infused intra-arterially [11]. Systemic L-NMMA raises blood pressure [12], but its effect upon insulin sensitivity is unknown. Other vasoactive agents (angiotensin II, norepinephrine and adrenoline) which raise blood pressure and increase skeletal muscle blood flow have disparate effects on insulin sensitivity [13–16]. We therefore examined whether the systemic inhibition of NO production by the substrate inhibitor of NO synthase, L-NMMA, altered whole-body glucose uptake.

METHODS

Subjects

Sixteen healthy male subjects, mean age 24.3±1.4 years, body mass index 22.8±0.6 kg/m² gave written informed consent to participate in this study which was approved by the Tayside Medical Ethics Committee. Two subjects smoked, both less than 10 cigarettes per day and were asked to refrain from smoking for 24 hours before the study. Six subjects were NIDDM (type 2) and were on multiple oral hypoglycaemic agents, and were taking no other medications. All subjects were non-smokers and had to have been regular (at least 20 cigarettes per day) smokers for at least one year to be included in the study. Subjects were assigned to treatment with randomization. Insulin (10 U/m²) was administered intravenously over a 60 minute period with a continuous infusion of glucose (0.5 mg/kg/min) and saline for 120 minutes thereafter in the control (saline) and treatment (L-NMMA) conditions. The insulin infusion was stopped 10 minutes before venous access was lost, and the last 10 minutes of glucose infusion were used as a baseline for comparison. The combination of glucose and saline used for the NO treatment was shown to have no effect on local or whole-body glucose uptake [17].

Glucose and insulin were injected intravenously to maintain a constant euglycaemic level of 5.0 mmol/l. Metabolic clearance rates of glucose and insulin were calculated from the area under the curves. Whole-body glucose uptake was measured by the stable isotope technique [18]. An insulin-resistant state was considered to be present if the whole-body glucose uptake achieved in the NO treatment was less than 80% of that achieved in the control condition.

1. Recent evidence shows that skeletal muscle blood flow is an important determinant of insulin sensitivity and that insulin-mediated vasodilatation is nitric oxide dependent. These results have given rise to the hypothesis that endothelial nitric oxide inhibition may decrease insulin sensitivity in humans.

2. We examined this hypothesis directly by evaluating the effects of systemic nitric oxide synthase inhibition with NG5-monomethyl L-arginine (3 mg h⁻¹ kg⁻¹) on whole-body glucose uptake (euglycaemic hyperinsulinaemic clamp) and calf blood flow (bilateral calf venous occlusion plethysmography) in 16 healthy male subjects in a randomized, double-blind, placebo-controlled, crossover study.

3. NG5-Monomethyl L-arginine infusion was associated with a pressor effect (119/61±2/2 mmHg for placebo; P<0.001), and a negative chronotropic response (57±2 beats/min for placebo; P<0.001). The glucose infusion rate was significantly increased after infusion of NG5-monomethyl L-arginine (8.9±0.9 compared with 7.9±0.8 mg min⁻¹ kg⁻¹ for placebo; P=0.002). Whole-body glucose uptake increased during the clamp, with values of 9.4±0.7 and 10.9±0.8 mg min⁻¹ kg⁻¹ for placebo and NG5-monomethyl L-arginine respectively (P=0.056; 95% confidence interval 0.2,2.8). NG5-Monomethyl L-arginine was associated with increased calf blood flow by comparison with placebo (P<0.05, area under curve).

4. These data show for the first time that systemic inhibition of nitric oxide synthesis increases rather than decreases whole-body glucose uptake. We suggest that the higher skeletal muscle blood flow seen after NG5-monomethyl L-arginine may explain the observed increase in whole-body glucose uptake.

Key words: blood flow, euglycaemic hyperinsulinaemic clamp, insulin sensitivity, NG5-monomethyl L-arginine, nitric oxide.

Abbreviations: ANOVA, analysis of variance; NIDDM, non-insulin-dependent diabetes mellitus; L-NMMA; NG5-monomethyl L-arginine; NO, nitric oxide.

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from tobacco. All subjects were asked to avoid strenuous exercise and none was receiving any medication.

**Study protocol**

After initial screening, each volunteer attended two 5 h study mornings, at least 1 week apart, to evaluate the effects of placebo and L-NMMA (3 mg h⁻¹ kg⁻¹) on whole-body glucose uptake in a randomized, double-blind, crossover design.

On each occasion, after a 12 h overnight fast (water was permitted), subjects attended a temperature-controlled room (23°C) in our research unit at 07.45 hours. After a 20 min supine rest, baseline blood pressure and heart rate measurements were recorded. A 18G cannula was inserted into the left antecubital fossa for administration of test substances and a 20G cannula was inserted retrogradely into the dorsum of the right hand for blood sampling. The right hand was then rested in a heated box (temperature 55°C) to arterialize the venous blood.

**Euglycaemic hyperinsulinaemic clamp.** On each study day, whole-body glucose uptake (M) was calculated during the last 40 min of a 3 h euglycaemic hyperinsulinaemic clamp as described previously [18]. A primed constant-rate infusion of soluble insulin (1.5 m-units min⁻¹ kg⁻¹) in 45 ml of saline (0.9% NaCl) with 5 ml of the patient's own blood (to prevent adsorption of insulin to the internal surface of the syringe) was administered for 3 h. At the same time, a variable rate infusion of 20% (wt/vol.) dextrose was administered (IVAC Pumps, San Diego, CA, U.S.A.) to maintain euglycaemia (5.0 mmol/l). A third constant-rate infusion of either saline or L-NMMA (3 mg h⁻¹ kg⁻¹) (Clin-Alfa AG, Switzerland) was administered for 180 min. L-NMMA or placebo was prepared by an independent investigator so that the main investigator and patient were blind to the nature of the infusate: this infuson was administered using a Braun perfusor pump. At 5 min intervals, 2 ml blood samples were collected for measurement of plasma glucose concentrations at the bedside (Beckman Instruments Inc., Fullerton, CA, U.S.A.).

Systolic and diastolic blood pressure and heart rate were measured using a Critikon Dinamap™ monitor at 15 min intervals during the clamp. At baseline and at 60 min intervals, additional blood samples were collected for measurements of serum insulin.

**Venous occlusion plethysmography.** Bilateral calf vein occlusion plethysmography [19] (Medasonics, Mountain View, CA, U.S.A.) was performed at baseline and then at 60 min intervals throughout the clamp. Pneumatic cuffs were placed around the ankle and inflated to 200 mmHg to isolate arterial circulation in the foot. Intermittently, a thigh cuff was inflated to 30 mmHg, to prevent venous drainage. The change in calf volume was estimated with mercury-filled strain gauges (stretched to calf circumference +20%). The mean of five measurements in each leg was taken and then averaged [19].

Blood samples for serum insulin (Incstar RIA, Minneapolis, U.S.A.; coefficient of variation 8.5%, inter-assay variation 1.4%) were frozen at –20°C and estimated in one batch.

**Statistical analysis**

Whole-body insulin sensitivity in mg glucose min⁻¹ kg⁻¹ (M) was calculated under steady-state conditions during the last 40 min of each clamp. M-values and blood flow measurements for individual subjects were compared between treatments by analysis of variance (ANOVA) and the Bonferroni method for calculating 95% confidence intervals, correcting for multiple comparisons. Results are expressed as means ± SE. Differences were considered statistically significant when P < 0.05.

**RESULTS**

The euglycaemic clamp and L-NMMA infusions were well tolerated. There were no adverse biochemical events and no withdrawals from the study.

**Blood pressure, heart rate and serum insulin measurements.**

L-NMMA was associated with a significant increase in systolic and diastolic blood pressure from 20 to 180 min (P < 0.001 by MANOVA); for example, mean blood pressure in the last 60 min of the clamp was 114/58 ± 2/2 mmHg for placebo compared with 119/61 ± 2/2 mmHg for L-NMMA. L-NMMA was associated with a significant reduction in supine heart rate (P < 0.001 by MANOVA); mean values in the last 60 min of the clamp were 63 ± 2 beats/min for placebo compared with 59 ± 2 beats/min for L-NMMA (Table 1).

Serum insulin levels increased to a plateau within the first 60 min of the infusion and there were no significant differences in insulin concentrations between the 2 study days (Fig. 1). For example, at 180 min, serum insulin concentrations were 137 ± 9 μ-units/ml and 142 ± 9 μ-units/ml after placebo and L-NMMA respectively (Table 2).

**Plasma glucose, glucose infusion rate and whole-body insulin sensitivity**

There was no significant difference in plasma glucose concentrations during the clamps. Mean plasma glucose concentrations for the last 40 min were 5.0 ± 0.05 and 5.0 ± 0.05 mmol/l. The coefficient of variation for plasma glucose concentration was less than 5% for all clamps (Table 2, Fig. 1).

The glucose infusion rate was significantly increased after infusion of NGO-monomethyl
Table 1. Baseline and hourly measurements during the clamp. Values are means ± SE. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate. *P < 0.001 L-NMMA versus placebo for SBP, DBP and HR (15–180 min).

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<th>Placebo</th>
<th>L-NMMA</th>
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<tr>
<td></td>
<td>SBP (mmHg)</td>
<td>DBP (mmHg)</td>
</tr>
<tr>
<td>Baseline</td>
<td>112.7 ± 3.0</td>
<td>56.9 ± 1.5</td>
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<tr>
<td>First hour</td>
<td>111.1 ± 2.2</td>
<td>57.2 ± 1.7</td>
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<tr>
<td>Second hour</td>
<td>111.8 ± 2.0</td>
<td>57.4 ± 1.8</td>
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<tr>
<td>Third hour</td>
<td>114.1 ± 1.9</td>
<td>58.4 ± 1.8</td>
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Table 2. Summary of clamp data from the last hour. Statistical significance: *P < 0.05; †P < 0.001.

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<th>Placebo</th>
<th>L-NMMA</th>
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<tbody>
<tr>
<td>Insulin (µ-units/ml)</td>
<td>137 ± 9</td>
<td>142 ± 9</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.0 ± 0.05</td>
<td>5.0 ± 0.05</td>
</tr>
<tr>
<td>Glucose infusion rate (mg min⁻¹ kg⁻¹) over last 20 min</td>
<td>9.8 ± 0.7†</td>
<td>11.0 ± 0.9†</td>
</tr>
<tr>
<td>Insulin sensitivity (mg min⁻¹ kg⁻¹), last 40 min</td>
<td>9.4 ± 0.7*</td>
<td>10.9 ± 0.8*</td>
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L-arginine (8.9 ± 0.9 compared with 7.9 ± 0.8 mg min⁻¹ kg⁻¹ for placebo; *P = 0.002) (Table 2, Fig. 1). L-NMMA increased insulin-stimulated glucose uptake; mean values for M were 9.4 ± 0.7 and 10.9 ± 0.8 mg min⁻¹ kg⁻¹ (*P = 0.036; 95% confidence interval 0.2, 2.8) for the placebo and L-NMMA infusion days respectively (Table 2, Fig. 2).

Calf blood flow

L-NMMA infusion was associated with a 30% increase (*P < 0.05, area under curve) in calf blood flow throughout the clamp compared with placebo; for example, at 180 min leg flow was 2.0 ± 0.2 ml min⁻¹ 100 ml⁻¹ and 1.6 ± 0.2 ml min⁻¹ 100 ml⁻¹ for L-NMMA and placebo respectively (Fig. 1).

DISCUSSION

The effect of systemic NO inhibition with L-NMMA on whole-body insulin sensitivity has not been described previously. The results demonstrate that in man in vivo, systemic L-NMMA was associated with a pressor response, an increase in whole-body glucose uptake and an increase in calf blood flow.

Methodological considerations

In our study we used the standard method for the measurement of insulin sensitivity, the hyperinsulinaemic euglycaemic clamp [18]. This technique calculates whole-body glucose uptake during a period of stable hyperinsulinaemia. It has some limitations; for example it fails to assess other insulin-mediated
responses which may be regulated maximally at lower insulin levels. It remains, however, a well-validated research tool [18] and whole-body glucose uptake is a valid surrogate for true insulin sensitivity. Our clamps were of good quality (coefficient of variation of plasma glucose <5%) and a reproducible hyperinsulinaemic stimulus was obtained on each study day.

Venous occlusion plethysmography is a valid and reproducible method for the assessment of limb blood flow [19–21]. Indeed, in the arm 50–75% of the total blood flow is through skeletal muscle [22] and in the leg the percentage is higher still. Therefore, a change in blood flow measured by plethysmography is likely to represent a significant change in skeletal muscle perfusion. The mean of the blood flow measurement in each calf was used to minimize experimental error [19].

Figure 1 demonstrates an apparent fall in blood flow during the placebo clamp which conflicts with some but not all previous work [3, 8, 9, 23, 24]. However, in our study there are no statistical differences between any of the time points on the placebo study day. Others have seen, like us, a small nonsignificant reduction in leg blood flow during hyperinsulinaemia alone [14]. In our case the apparent fall may be due to one volunteer who had high baseline values on both treatment and placebo days. There was no legitimate reason to exclude him and therefore his data is included in the analysis and figure. This odd finding, however, has no bearing on our main finding.

The dose of systemic l-NMMA used (3 mg h⁻¹ kg⁻¹) was guided by previous work which reported a significant increase in blood pressure and total peripheral vascular resistance [12] similar to that observed in our study. Previous work from this laboratory has shown that plasma nitrate, the major plasma metabolite of NO, falls by 30% (30.7 μmol/l to 21.5 μmol/l) after l-NMMA, 3 mg h⁻¹ kg⁻¹ [24a], indicating a decline in NO production, albeit under basal, non-insulin-stimulated conditions. Other data [25] suggest that this dose may be at the lower end of the dose–response range. However, there are two points in our study which show that a significant degree of overall NO blockade was achieved: firstly the increase in blood pressure and fall in heart rate and secondly the finding that this dose reduces plasma nitrate. There are no precise methods available for use in man to quantify exactly the degree of NO blockade produced in this or any experiment with l-NMMA in man.

The only previous reports on the metabolic effects of systemic NO inhibition are in animals. The results are conflicting; for example, in rats there are reports that oral ingestion of the competitive NO synthase inhibitor N⁶-nitro-l-arginine methyl ester induced hypertension but no alteration in glucose tolerance or insulin sensitivity [26], whereas acute intravenous infusion of l-NMMA in rats has been reported to produce hypertension and a reduction in insulin sensitivity [27]. In neither was skeletal muscle blood flow measured and species differences are likely to be a major confounding problem.

Existing data [4, 8–10] in man are confined to local intra-arterial infusion of l-NMMA. Contrary to our findings, Baron et al. [11] showed that the local infusion of l-NMMA decreased leg glucose uptake by 21%. There is evidence that increases in blood flow increase glucose uptake despite stable ambient plasma glucose and insulin concentrations. This has resulted in the hypothesis that skeletal muscle perfusion is an independent determinant of insulin-mediated glucose uptake [7]. Nuutila et al. [28] have recently challenged this view with an elegant series of experiments which utilized intra-

Fig. 2. Mean profiles of glucose infusion rate, insulin sensitivity (M) and individual M-values. Statistical significance: *P<0.05; †P<0.001.
arterial infusions of the vasodilator bradykinin and positron emission tomography to measure blood flow and glucose extraction, and concluded that increases in skeletal muscle blood flow were not per se associated with a concomitant increase in glucose uptake. Despite this it is still generally thought that, during euglycaemic hyperinsulinaemia, skeletal muscle is the main determinant of insulin sensitivity, which may account for 80% of total glucose uptake [3]. This view is given added credence by the fact that the effect of hyperinsulinaemia in increasing skeletal muscle blood flow is blunted in insulin-resistant states such as obesity, NIDDM and hypertension [3, 5, 6]. It is still generally thought that insulin-induced vasodilatation is NO dependent, despite some controversy [3, 8, 9, 23, 24].

It is pertinent to compare the effects that we observed with systemic L-NMMA with other systemically administered vasoactive agents. Indeed, characterizing the pharmacological effects of L-NMMA in vivo, in whole man, was a specific aim of our study as each pharmacological substance behaves differently (as described below). For example, angiotensin II and noradrenaline [29] both cause profound vasoconstriction when infused intra-arterially, but when administered systemically they both increase skeletal muscle blood flow [16] and insulin sensitivity [14, 30] in a manner analogous to our findings with L-NMMA, although some conflicting data exist for noradrenaline [19]. It is important to realize, however, that all pharmacological agents that increase muscle blood flow do not automatically increase insulin sensitivity. Such effects do not occur with adrenaline; for example, Deibert et al. [15] infused adrenaline systemically during a hyperinsulinaemic euglycaemic clamp and reported that insulin sensitivity reduced by 50% when compared with a sham clamp. It is well known that adrenaline increases systolic blood pressure, increases skeletal muscle blood flow but conversely appears to decrease insulin sensitivity. Therefore, the adrenaline data means that we cannot assume that our findings with L-NMMA (16% increase in insulin sensitivity and 30% increase in calf blood flow) are necessarily due to haemodynamic changes alone, since adrenaline shows divergent effects on muscle blood flow and insulin sensitivity. However, we feel that adrenaline is uniquely different and that the effects we have seen with L-NMMA are due to haemodynamic factors which makes L-NMMA similar in its behaviour to angiotensin II and noradrenaline.

The ultimate effect of vasoactive compounds on insulin-mediated responses is probably dependent on whether local pharmacological effects or systemic haemodynamic effects predominate. A complex picture arises in the balance between these two effects and each pharmacological substance should be characterized individually (Table 3). The main purpose of our study was to characterize L-NMMA’s effects in this regard in man in vivo, and to see if it behaved in accord with angiotensin II and noradrenaline or in accord with adrenaline.

One other explanation for our findings is that different vascular beds have different sensitivities to NO. Although we did not measure blood flow in different vascular beds, previous work has shown that constitutive NO synthase activity is lower in conduit vessels and higher in resistance vessels [31]. An extension of this idea of differential sensitivity is the possibility that at low doses of L-NMMA, vasoconstriction in non-skeletal muscle vascular beds diverts blood to skeletal muscles, but if the dose is increased, vasoconstriction will begin to exert itself in the muscular bed leading to decreased insulin sensitivity. Several factors suggest this is unlikely but not impossible: firstly, the systemic dose of L-NMMA infused is equivalent to intra-arterial doses known to cause vasoconstriction in the human forearm. Secondly, circumstantial evidence from other vasoconstrictors, angiotensin II [16] and noradrenaline [13], suggests that increasing the dose of the vasoactive agent merely increases the observed effect on blood flow. Thirdly, there is no previous example of which we are aware of a pharmacological substance producing opposite effects at high dose and low dose. Finally, as the dose of L-NMMA is increased, the likely effect is that even more blood will be diverted to the skeletal muscle vascular bed as the non-muscle beds are likely to vasoconstrict even more than the muscular beds, i.e. at a high dose of L-NMMA, the diversion to muscle beds is likely to be accentuated rather than attenuated.

In view of the structural similarity between L-NMMA and L-arginine, one possible explanation might have been that L-NMMA stimulated insulin

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<th>Insulin sensitivity</th>
<th>Muscle blood flow</th>
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<tr>
<td>L-NMMA</td>
<td>EC</td>
<td>16% increase compared with placebo</td>
<td>30% increase compared with placebo</td>
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<tr>
<td>Angiotensin II</td>
<td>EC</td>
<td>33% increase compared with placebo</td>
<td>56% increase in femoral artery blood flow compared with placebo</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>EC</td>
<td>17% increase compared with placebo</td>
<td>55% increase in femoral artery blood flow compared with placebo</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>EC</td>
<td>50% reduction compared with placebo</td>
<td>Not measured</td>
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release. Work in animals suggests that N\textsuperscript{G}-nitro-L-arginine methyl ester does increase insulin secretion, but that this effect is banished by fasting [32]. However, our observed change in insulin sensitivity is unlikely to be explained by a direct effect of L-NMMA on insulin release or clearance, because of the close approximation of steady-state plasma insulin concentrations on each study day.

The other explanation for our findings worth considering is a direct biochemical effect of L-NMMA in enhancing glucose transport. Limited in vitro data make this latter possibility unlikely since NO tends to give the opposite result [33]. Alternative possibilities are that the increase in whole-body glucose uptake could have occurred in insulin-insensitive tissue or have been non-insulin-mediated in insulin-sensitive tissue. Our present study did not measure hepatic glucose output during the clamp and therefore cannot exclude a direct effect of L-NMMA on hepatic glucose production.

CONCLUSION

Systemic L-NMMA increased whole-body glucose uptake. The precise mechanism for these observations will require further investigation but the higher skeletal muscle blood flow seen with L-NMMA seems the likely explanation for our observed increase in whole-body glucose uptake. Our results raise the possibility that different vascular beds may display differential sensitivity to NO. Further studies will be required to clarify this issue.

ACKNOWLEDGMENTS

R.B. is supported by a project grant from the British Heart Foundation. This study was also supported by equipment grants from Tenvous Tayside, a local Anonymous Trust and the Nuffield Foundation. We thank Mrs Wendy Coutie for her excellent technical assistance.

REFERENCES