NO-dependent and -independent elevation of plasma levels of insulin and glucose in rats by L-arginine

T. Jun & ¹Å. Wennmalm

Division of Clinical Physiology, Göteborg University, Sahlgrenska Hospital, S-413 45 Göteborg, Sweden

1 L-Arginine elevates plasma insulin in man. Recent *in vitro* data indicate that this is based on stimulation of endogenous nitric oxide (NO) with subsequent pancreatic release of insulin by L-arginine. L-Arginine also raises plasma glucose.

2 We studied plasma levels of insulin, glucose and NO metabolites, as well as systemic blood pressure, in anaesthetized rats during i.v. infusion of L-arginine (25–200 mg kg⁻¹ min⁻¹) or glucose (55 mg kg⁻¹ min⁻¹), before and after administration of the NO synthesis inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME, 50 mg kg⁻¹).

3 Before L-NAME, L-arginine elevated plasma insulin from about 15 to 65 u l⁻¹ and glucose from 5.2 to 6.7 mmol l⁻¹. These effects of L-arginine were not dose-related.

4 L-NAME alone had no effect on plasma insulin and glucose levels, but diminished the effects of a low dose (25 mg kg⁻¹ min⁻¹) of L-arginine on plasma insulin by about 40%, and that on plasma glucose by more than 90%. In contrast, the effects of a high dose (200 mg kg⁻¹ min⁻¹) of L-arginine on plasma insulin and glucose levels were not affected by L-NAME.

5 L-NAME elevated systemic blood pressure by about 35 mmHg. L-Arginine (25–100 mg kg⁻¹ min⁻¹) had no effect on systemic blood pressure, either before or after L-NAME. L-Arginine (200 mg kg⁻¹ min⁻¹) lowered systemic blood pressure, both before and after L-NAME.

6 Glucose infusion elevated plasma glucose from about 5.5 to 6.8 mmol l⁻¹, and plasma insulin from about 18 to 26 u l⁻¹.

7 The basal plasma levels of the NO metabolite nitrate (18 ± 4 µmol l⁻¹) were not affected by L-arginine (200 mg kg⁻¹ min⁻¹). Plasma nitrosohaemoglobin was likewise unaffected by L-arginine (200 mg kg⁻¹ min⁻¹).

8 We conclude that L-arginine separately elevates plasma insulin and glucose levels, both by NO-dependent and -independent mechanisms.

Keywords: Anaesthetized rat; L-arginine; blood pressure; glucose; insulin; nitrate; nitric oxide

Introduction

Nitric oxide (NO), formerly known as endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), is formed when the amino acid, L-arginine, enzymatically incorporates molecular oxygen and converts it to L-citrulline (Palmer *et al.*, 1988). NO is a biomediator with widespread biological actions in tissues (Moncada *et al.*, 1991).

L-Arginine, when given intravenously, stimulated insulin release in man (Floyd *et al.*, 1966); it also had a glucogenic action. The latter effect was, however, moderate and considered not responsible for the increase in plasma insulin (Floyd *et al.*, 1966).

Recently it was demonstrated that insulin release stimulated by L-arginine in the presence of D-glucose required an intact formation of nitric oxides (NOs) (Schmidt *et al.*, 1992). The assumption was based on evidence obtained in anaesthetized rats, isolated islets of Langerhans from rats, and a glucose-responsive clonal pancreatic β cell line (Schmidt *et al.*, 1992).

To explore further the possible role of endogenously formed NO in the release of insulin, we infused different doses of L-arginine in rats, before and following pretreatment with the NO synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME). Plasma levels of insulin, glucose and nitrate (metabolite of NO) were followed at intervals and blood pressure was recorded continuously. The plasma insulin response to direct infusions of glucose was also assessed.

Methods

Experimental procedures

Male Wistar rats, weighing 200–250 g, were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, i.p.) and tracheotomized. A polyethylene catheter flushed with heparin was inserted into the right carotid artery, and connected via a pressure transducer (Harvard Apparatus, Millis, MA, U.S.A.) to a polygraph (Model 7D, Grass Instruments, Quincy, MA, U.S.A.) for continuous recording of systemic blood pressure. Heart rate was recorded from the blood pressure tracing. Another catheter was inserted into the right femoral vein, for administration of infusion solution or drug. A third catheter was inserted into the right femoral artery and used for blood sampling. After insertion of catheters the rats were allowed to stabilize for at least 10 min before the experiment was started.

Forty non-fasted rats, divided into four groups, were studied. One group was given L-arginine (free base, Sigma Chemical Co., St. Louis, MO, U.S.A.; 25, 100, or 200 mg kg⁻¹ min⁻¹) at infusion rates of 0.1, 0.1 and 0.2 ml min⁻¹, respectively. The second group was pretreated with L-NAME (Sigma, 50 mg kg⁻¹, given i.v. during 5 min) 8–10 min prior to administration of L-arginine as above. Each rat was given one infusion only, and each infusion of L-arginine was maintained for 5 min. Blood samples for analysis of insulin and glucose were drawn before and at the end of the 5 min infusion of L-arginine. The third group was given glucose i.v. (55 mg kg⁻¹ min⁻¹) for 5 min, and blood samples were taken as above. The fourth group was given L-arginine (200 mg kg⁻¹ min⁻¹) as above, and blood samples were taken before

¹ Author for correspondence.

and at the end of the 5 min infusion of L-arginine for measurement of plasma nitrate. Blood pressure was recorded continuously in all groups throughout the experiments.

Analyses

Plasma insulin was measured by a clinical routine assay (Insulin radioimmunoassay kit, Pharmacia Diagnostics, Uppsala, Sweden). Plasma glucose was measured by an enzyme membrane analyser (Eppendorff).

Plasma nitrate was determined with a stable isotope ($\text{Na}^{15}\text{NO}_3$) dilution assay developed in our laboratory, utilizing positive ion/chemical ionization gas chromatography/mass spectrometry, after conversion of endogenous and labelled nitrate in the samples to nitrotoluene. The method has been described in detail elsewhere (Wennmalm *et al.*, 1993).

Analysis of nitroso-haemoglobin (HbNO) was performed as described previously (Wennmalm *et al.*, 1990). Samples of whole blood were rapidly centrifuged, followed by freezing of the cell fraction in quartz tubes in liquid nitrogen. Electron paramagnetic resonance (EPR) spectra of the samples were recorded at 77K in a Varian EPR spectrometer (Varian, Palo Alto, CA, U.S.A.) at a microwave frequency of 9.24 Hz and a microwave power of 10 mW. Spectra were scanned from 3.000 to 3.500 gauss with a modulation amplitude of 10 gauss and a scan rate of 25 gauss cm^{-1} .

Calculations

Differences between groups were analysed by Student's *t* test (two-tailed) for paired or unpaired observations, as appropriate. A *P* value <0.05 was considered significant. Data are presented as mean $\pm$ standard error (s.e.).

Results

Effect of L-arginine on plasma insulin and glucose, before and after L-NAME

The basal plasma levels of insulin and glucose in the anaesthetized rats, prior to infusion of L-arginine, were 14.6 ± 0.7 ($n = 15$) u l^{-1} and 5.24 ± 0.23 ($n = 15$) mmol l^{-1} , respectively.

Infusion of L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$) for 5 min increased plasma insulin to $31.4 \pm 27 \text{ u l}^{-1}$ ($P < 0.001$; $n = 5$), and plasma glucose to $7.04 \pm 0.10 \text{ mmol l}^{-1}$ ($P < 0.001$; $n = 5$). Infusion of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) for 5 min elevated plasma insulin to $65.3 \pm 7.2 \text{ u l}^{-1}$ ($P < 0.01$; $n = 5$), and plasma glucose to 6.65 ± 0.11 ($P < 0.001$; $n = 5$) mmol l^{-1} . L-Arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) increased plasma insulin to $52.2 \pm 3.1 \text{ u l}^{-1}$ ($P < 0.001$; $n = 5$), and plasma glucose to $6.58 \pm 0.15 \text{ mmol l}^{-1}$ ($P < 0.001$; $n = 5$). The increases in plasma insulin and glucose levels are shown in Figure 1.

Pretreatment with L-NAME (50 mg kg^{-1}) did not affect the basal plasma concentrations of insulin or glucose, the levels reached after drug treatment being 13.9 ± 0.8 ($n = 15$) u l^{-1} , and 5.43 ± 0.12 ($n = 15$) mmol l^{-1} . The plasma insulin and glucose responses to infusion of L-arginine were, however, diminished. Following pretreatment with L-NAME, L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$) elevated plasma insulin to 23.0 ± 2.7 ($P < 0.001$; $n = 5$) u l^{-1} . The difference in plasma insulin levels following infusion of L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$), before and after pretreatment with L-NAME, is of borderline significance ($P = 0.056$). Plasma glucose was not significantly affected by L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$) after pretreatment with L-NAME. The difference in plasma glucose levels following infusion of L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$), before and after pretreatment with L-NAME, is therefore highly significant ($P < 0.001$).

The plasma insulin and glucose responses to L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) were also diminished by L-NAME. Plasma insulin rose to 45.4 ± 3.6 ($P < 0.01$; $n = 5$) u l^{-1} , and

plasma glucose increased to 5.99 ± 0.18 ($P < 0.01$; $n = 5$) mmol l^{-1} . Both the insulin and glucose levels following infusion of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) were lower ($P < 0.05$ for both) after compared to before L-NAME. Infusion of L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) increased plasma insulin to 51.3 ± 3.0 ($P < 0.001$; $n = 5$) u l^{-1} , and plasma glucose to 6.51 ± 0.40 ($P < 0.05$; $n = 5$) mmol l^{-1} . These levels do not differ from the plasma insulin and glucose levels obtained following infusion of L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) in the absence of L-NAME.

The increases in plasma insulin and glucose levels in response to infusion of L-arginine ($25\text{--}200 \text{ mg kg}^{-1} \text{ min}^{-1}$) following pretreatment with L-NAME are shown in Figure 1.

Effect of L-arginine on systemic blood pressure before and after L-NAME

Basal heart rate and mean systemic blood pressure in the anaesthetized rats, prior to infusion of L-arginine, were 403 ± 10 ($n = 15$) beats min^{-1} and 111 ± 3 ($n = 15$) mmHg , respectively. Infusion of L-arginine (25 or $100 \text{ mg kg}^{-1} \text{ min}^{-1}$) did not affect heart rate or systemic blood pressure, while L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) lowered heart rate to 364 ± 11 ($P < 0.05$; $n = 5$) beats min^{-1} , and systemic blood pressure to 70 ± 6 ($P < 0.001$; $n = 5$) mmHg .

Infusion of L-NAME ($50 \text{ mg kg}^{-1} \text{ min}^{-1}$) decreased the heart rate to 348 ± 7 ($P < 0.001$; $n = 15$) beats min^{-1} , and elevated systemic blood pressure to 140 ± 2 ($P < 0.001$; $n = 15$) mmHg . Infusion of L-arginine (25 or $100 \text{ mg kg}^{-1} \text{ min}^{-1}$) after pretreatment with L-NAME failed to affect heart rate or systemic blood pressure, while L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) lowered heart rate to 324 ± 7 ($P < 0.05$; $n = 5$) beats min^{-1} , and systemic blood pressure to 86 ± 9 ($P < 0.01$, $n = 5$) mmHg .

Effect of infusion of glucose on plasma levels of insulin and glucose

Infusion of glucose ($55 \text{ mg kg}^{-1} \text{ min}^{-1}$) for 5 min elevated plasma insulin from 18.1 ± 0.8 ($n = 5$) IR u ml^{-1} to 26.2 ± 2.6 IR u ml^{-1} ($P < 0.05$). In parallel, the plasma concentration of glucose rose, from 5.49 ± 0.17 ($n = 5$) to 6.82 ± 0.25 mmol l^{-1} ($P < 0.01$). The plasma glucose and insulin levels

Figure 1 Increases in plasma insulin (a) and glucose (b) in rats after 5 min of infusion of L-arginine at the doses indicated. Open columns: no pretreatment. Hatched columns: animals pretreated with N^G -nitro-L-arginine methyl ester (L-NAME, 50 mg kg^{-1}). Columns show mean with s.e. ($n = 5$).

Figure 2 Increases in plasma glucose and insulin in rats after 5 min of infusion of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) or glucose ($55 \text{ mg kg}^{-1} \text{ min}^{-1}$). Solid columns: infusion of L-arginine. Hatched columns: infusion of glucose. Columns show mean with s.e. ($n = 5$).

after infusion of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) or of glucose ($55 \text{ mg kg}^{-1} \text{ min}^{-1}$) are shown in Figure 2. Glucose infusion did not affect the systemic blood pressure (120 ± 6 and $121 \pm 7 \text{ mmHg}$ before and after, respectively, the infusion).

Effect of L-arginine on plasma nitrate and red blood cell HbNO

The basal plasma level of nitrate was 18.3 ± 3.6 ($n = 5$) $\mu\text{mol l}^{-1}$. EPR spectrometry revealed no significant amounts of HbNO in the red blood cells. Following infusion of L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) plasma nitrate was $16.9 \pm 2.6 \mu\text{mol min}^{-1}$. The difference compared to before infusion is not significant. EPR spectrometry did not display any significant effect of infusion of L-arginine on red blood cell levels of HbNO.

Discussion

Infusion of L-arginine ($25\text{--}200 \text{ mg kg}^{-1} \text{ min}^{-1}$) caused a marked increase in plasma insulin, and a moderate elevation of plasma glucose in the present study. Neither of these effects of L-arginine was dose-dependent. After administration of the NO synthase inhibitor, L-NAME, the effects of L-arginine ($25\text{--}100 \text{ mg kg}^{-1} \text{ min}^{-1}$) on plasma insulin and glucose were diminished, while the effect of L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) was unaffected. Also, after L-NAME the effects of L-arginine on plasma insulin and glucose levels became dose-dependent. L-Arginine had no effects on plasma levels of NO metabolites, and failed to affect NO-dependent regulation of systemic blood pressure.

The presently observed effect of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$) on plasma insulin concentration was more marked than that of L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$), suggesting a dose-dependence of the effect in the interval $25\text{--}100 \text{ mg kg}^{-1} \text{ min}^{-1}$. However, at a dose of $200 \text{ mg kg}^{-1} \text{ min}^{-1}$, the effect of L-arginine on plasma insulin dropped somewhat, in comparison to that obtained at $100 \text{ mg kg}^{-1} \text{ min}^{-1}$. Schmidt *et al.* (1992) reported that in pancreatic β -cells with normal islet insulin secretory response, L-arginine elicited insulin release with an EC_{50} of $150 \mu\text{M}$; above this concentration L-arginine was less effective. The present data are consistent with a similar type of dose-dependence *in vivo*. Hence, it appears that L-arginine also stimulates pancreatic secretion of insulin in intact animals.

In parallel to its stimulatory effect on insulin secretion, L-arginine elevated plasma glucose. This effect also failed to present any clear dose-dependence. L-Arginine is, like several other amino acids, glucogenic. The proposed mechanism is that L-arginine is converted to glutamate, which is transaminated to form α -glutarate and, after entering the citric acid cycle, is metabolised or increases the glucose level (Orten & Neuhaus, 1975). The parallel increases in insulin and glucose induced by L-arginine might be taken to indicate

that the insulin response was secondary to the glucogenic effect of L-arginine. The experiments in which glucose was infused made it clear, however, that the increases in plasma glucose could not account solely for the elevation of plasma insulin. Infusion of glucose increased plasma glucose at least as much as L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$), but the resulting increase in plasma insulin was much smaller during the infusion of glucose than during infusion of L-arginine ($100 \text{ mg kg}^{-1} \text{ min}^{-1}$).

L-NAME is an efficient inhibitor of the constitutive NO synthase (Rees *et al.*, 1990). In accordance with previous observations (Gardiner *et al.*, 1990), administration of L-NAME (50 mg kg^{-1}) caused a marked increase in mean arterial blood pressure in the rats presently studied, indicating that endothelial NOS was inhibited. L-Arginine ($25\text{--}100 \text{ mg kg}^{-1} \text{ min}^{-1}$) was unable to lower the blood pressure after L-NAME, suggesting that the inhibition of NOS could not be reversed by a large increase in substrate availability. The highest dose of L-arginine ($200 \text{ mg kg}^{-1} \text{ min}^{-1}$) did lower the blood pressure quite substantially. However, we do not interpret this effect of the amino acid as based on an increased endothelial formation of NO. The reason for this is that a similar effect of L-arginine was observed also in the absence of L-NAME. The assumption of an unspecific, i.e. not related to NO formation, effect of L-arginine is further supported by observations that both L-arginine and D-arginine (the latter not being substrate for NO synthase) act as vasodilators in human superficial veins and resistance vessels *in vivo* (Calver *et al.*, 1991).

L-NAME did not affect the basal plasma levels of insulin or glucose, suggesting that the secretion of insulin and the regulation of glucose homeostasis in the basal state are not under influence of NO. However, after L-NAME the stimulatory effect of L-arginine on plasma insulin and glucose levels changed both qualitatively and quantitatively. The effect of L-arginine, both on plasma insulin and glucose levels, became clearly dose-related. The lowest dose of L-arginine, i.e. $25 \text{ mg kg}^{-1} \text{ min}^{-1}$, was less potent in stimulating plasma insulin, and furthermore, was no longer able to elevate plasma glucose. In contrast, the highest dose of L-arginine, $200 \text{ mg kg}^{-1} \text{ min}^{-1}$, was equally efficient in raising both plasma insulin and glucose levels before and after L-NAME.

The data available are not sufficient to interpret conclusively the mechanism behind the change in response pattern caused by L-NAME. One possibility would be that L-NAME did inhibit NOS at the sites of secretion of insulin and glucose, and that the remaining responses after NOS inhibition displayed effects of L-arginine on insulin and glucose secretion that are not NO-dependent. Another possibility would be that the NOS at the sites of insulin and glucose secretion had other kinetic properties than endothelial NOS (which was probably inhibited), and that the increasing doses of L-arginine given were able to break through progressively the inhibition caused by L-NAME. A third possibility would be that after L-NAME, the direct stimulatory effect of L-arginine on glucose secretion was sufficient to cause the observed elevation of plasma insulin. However, this alternative is not entirely likely inasmuch as plasma insulin, after L-NAME, was significantly increased by the lowest dose of L-arginine ($25 \text{ mg kg}^{-1} \text{ min}^{-1}$) while plasma glucose was not.

In addition to these alternative explanations, others must be considered as well. Despite the uncertainty in the interpretation of the results, the data obtained seem to merit the following conclusions. L-Arginine elevated plasma insulin and glucose by mechanisms that were independent of each other, i.e. the insulin response to L-arginine could not be entirely due to the glucogenic effect of the amino acid. Furthermore, both the insulin and glucose effects of L-arginine were partly blocked by L-NAME, indicating that NO formation participated to a certain extent both in the insulin and glucose secretory responses to L-arginine.

The extent to which the observed NO-dependent mechanisms for insulin and glucose secretion *in vivo* bear any relation to the impairment of glucose homeostasis in diabetes requires further studies. Infusion of L-arginine is a frequently used tool for stimulation of insulin secretion *in vivo*. The potentiating effect of arginine on the secretion of insulin in response to glucose is decreased in patients with non-insulin

dependent diabetes mellitus (Ward *et al.*, 1984). The present data call for studies to reveal if such a decrease is related to an impaired formation of NO.

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