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Alterations of insulin response to different β cell secretagogues and pancreatic vascular resistance induced by N$^{\omega}$-nitro-L-arginine methyl ester

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1 We studied a possible interplay of pancreatic NO synthase activity on insulin secretion induced by different β cell secretagogues and also on pancreatic vascular bed resistance.

2 This study was performed in the isolated perfused pancreas of the rat. Blockade of NO synthase was achieved with N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME); the specificity of the antagonist was checked by using its D-enantiomer as well as by substitutive treatments with sodium nitroprusside (SNP) as a NO donor in studies of glucose-induced insulin secretion.

3 Arginine (5 mM) induced a monophasic insulin response which was, in the presence of L-NAME at equimolar concentration, very strongly potentiated and converted into a 13 times higher biphasic one. D-NAME (5 mM) was only able to induce a 3 times higher response, but provoked a similar vasoconstrictor effect.

4 The small biphasic insulin secretion induced by L-leucine (5 mM) was also strongly enhanced, by 8 times, in the presence of L-NAME (5 mM) vs 2 times in the presence of D-NAME (5 mM).

5 β cell responses to KCl (5 mM) and tolbutamide (0.185 mM) were only slightly increased by L-NAME (5 mM) to values not far from the sum of the effects of L-NAME and of the two drugs alone. D-NAME (5 mM) was totally ineffective on the actions of both secretagogues.

6 L-NAME, infused 15 min before and during a rise in glucose concentration from 5 to 11 mM, was able in the low millimolar range (0.1–0.5 mM) to blunt the classical biphasic pattern of β cell response to glucose and, at 5 mM, to convert it into a significantly greater monophasic one. In contrast, D-NAME (5 mM) was unable to induce similar effects.

7 SNP alone at 3 μM was ineffective but at 30 μM substantially reduced the second phase of insulin response to glucose; however, at both concentrations the NO donor partly reversed alterations in insulin secretion caused by L-NAME (5 mM) and restored a biphasic pattern of insulin response. At a high (300 μM) concentration, SNP drastically reduced the second phase of β cell response, but in the presence of L-NAME, provoked a significantly greater biphasic response.

8 When L-NAME was infused only for the 15 min before high glucose, an exaggerated first phase of β cell response was followed by an abortive second one. SNP, at a low concentration (30 nM), given simultaneously with L-NAME, restored a biphasic pattern and prevented the vasoconstrictor effect induced by the inhibitor.

9 L-NAME, when infused only during high glucose, markedly enhanced the second phase of insulin response which could be significantly reduced by SNP (3 μM). The NO donor induced a dilator effect significantly greater in L-NAME-treated pancreata than in non-treated ones.

10 In conclusion our data bring evidence that NO synthase activity exerts an inhibitory control on pancreatic β cell response to various nutrient secretagogues and may, at least partly, be implicated in the generation of the biphasic pattern of insulin response to glucose.

Keywords: Insulin secretion; glucose; arginine; leucine; pancreatic vascular resistance; nitric oxide synthase inhibitor, N$^{\omega}$-nitro-L-arginine methyl ester; nitric oxide donor; sodium nitroprusside

Introduction

Nitric oxide (NO) is a new messenger molecule implicated in a number of physiological functions such as vascular tone (Palmer *et al.*, 1988) platelet aggregation (Radomski *et al.*, 1990) and inhibitory neurotransmission (Bult *et al.*, 1990), and pathological ones, among which is the cytotoxicity of activated macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988). This short-lived oxygen free radical is synthesized from the terminal guanidino nitrogen atoms of L-arginine (Palmer *et al.*, 1988; Hibbs *et al.*, 1988; Marletta *et al.*, 1988) by isoforms of NO synthase, purified and cloned from different tissues (Knowles & Moncada, 1994) and which are of at least two different types. One is constitutive and Ca^{2+}/calmodulin-dependent and the second inducible by cytokines and Ca^{2+} insensitive. The physiological effects of NO are accounted for by small amounts produced by the Ca^{2+} -dependent isoform, whereas cytotoxic effects result from considerably larger amounts of NO generated immunologically by the inducible form of NO synthase (Moncada *et al.*, 1991a).

With regard to pancreatic function, insulin-dependent diabetes is an autoimmune disease (Gepts, 1965) characterized by lymphocytic infiltration into pancreatic islets, and NO has been implicated as the effector molecule that mediates cytokine-induced inhibitory and cytotoxic effects on rat (Southern *et al.*, 1990) and human (Corbett *et al.*, 1993) islets. The Ca^{2+}-dependent constitutive isoform of NO synthase is present in islets (Laychock *et al.*, 1991; Schmidt *et al.*, 1992). However conflicting results have been reported as to its activation by β cell secretagogues; indeed arginine and glucose have been

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shown either to increase NO production in transformed insulin secreting cell lines (Schmidt *et al.*, 1992) or to be ineffective in cultured islets (Jones *et al.*, 1992). Whether such a discrepancy results from the different experimental models and/or assays used remains to be determined. So, using a different approach, we were interested in investigating whether NO synthase activity could interfere with the secretory effect of different β cell secretagogues. First, we used arginine, the physiological substrate of the enzyme, but also glucose and leucine which are able to induce rapid increases in intracellular levels of NADPH and Ca^{2+}, two important co-factors for the enzyme activity (Moncada *et al.*, 1989). KCl and tolbutamide, two non metabolic stimuli, were then used to investigate whether NO synthase activity might differently affect insulin secretion induced either by metabolic secretagogues or by agents acting primarily through ionic events. The effects of L-NAME acting specifically through a decrease in NO production were tested by comparing its effects with those of its D-enantiomer on the different secretagogues-induced insulin secretion; furthermore with respect to the β cell physiological stimulus, glucose, we also used SNP as a palliative NO donor in attempt to counteract L-NAME-related alterations. Our work was carried out in the isolated perfused pancreas of the rat which preserves the anatomical integrity of islets and pancreatic vascularization, thereby allowing simultaneous study of secretory effects and changes in vascular resistance.

Methods

For this study we used adult male Wistar rats (Iffa-Credo, Lyon, France) weighing 340–380 g; they were fed a standard pellet diet (U.A.R., Epinay-sur-Orge, France) *ad libitum* and had free access to tap water. The rat pancreata were isolated and perfused as previously described (Loubatières *et al.*, 1969). Briefly, after sodium pentobarbitone anaesthesia, the pancreas was completely isolated from all neighbouring tissues, placed in a thermostatted (37.5°C) plastic chamber, and perfused via an open circuit through its own arterial system. The perfusion

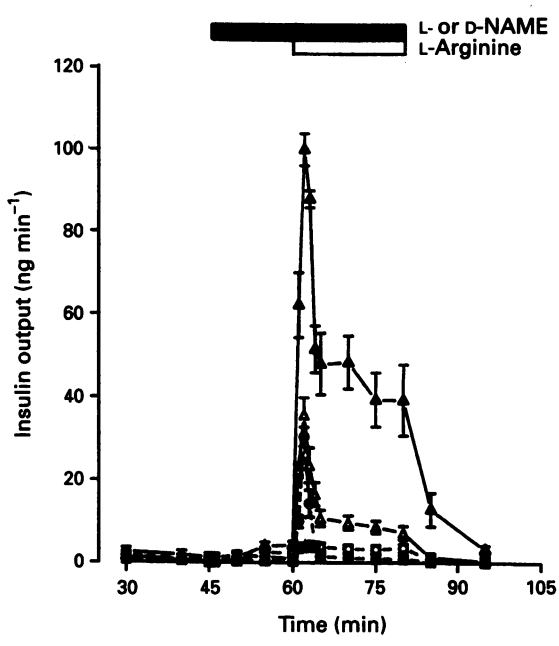

Figure 1 Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 5 mM) or D-NAME (5 mM) on arginine (5 mM)-induced insulin secretion. Traces show arginine alone (●); L-NAME alone (○); arginine + L-NAME (▲); arginine + D-NAME (△). Points are means ± s.e.mean from 5–7 experiments. The perfusion medium contained 5 mM glucose throughout; arginine and L- or D-NAME were present for the periods shown by the horizontal bars.

Table 1 Effect of L or D-NAME on rat pancreatic vascular resistance in presence or absence of different secretagogues

					L or D-NAME					
					Secretagogue					
Min	*30*	*45*	*50*	*55*	*60*	*65*	*70*	*75*	*80*	*95*
L-NAME 5 mM	2.48 ±0.02	2.47 ±0.02	2.27 ±0.05	2.08 ±0.04	1.96 ±0.02	1.83 ±0.03	1.79 ±0.03	1.73 ±0.03	1.67 ±0.04	1.58 ±0.10
D-NAME (5 mM)	2.50 ±0.02	2.47 ±0.02	2.35 ±0.03	2.12 ±0.05	2.03 ±0.05	2.01 ±0.04	1.91 ±0.07	1.85 ±0.09	1.84 ±0.08	1.69 ±0.09
L-Arginine 5 mM	2.49 ±0.02	2.46 ±0.02	2.43 ±0.03	2.43 ±0.03	2.46 ±0.03	2.69 ±0.04	2.66 ±0.02	2.68 ±0.05	2.67 ±0.05	2.36 ±0.03
L-Arginine 5 mM + L-NAME 5 mM	2.50 ±0.00	2.48 ±0.01	2.26 ±0.03	2.16 ±0.05	1.96 ±0.05	1.94 ±0.05	1.93 ±0.04	1.90 ±0.03	1.83 ±0.03	1.62 ±0.04
L-Arginine 5 mM + D-NAME 5 mM	2.44 ±0.07	2.41 ±0.03	2.19 ±0.03	2.04 ±0.07	2.01 ±0.09	2.00 ±0.11	2.03 ±0.09	2.01 ±0.09	2.03 ±0.10	1.90 ±0.11
L-Leucine 5 mM	2.49 ±0.01	2.40 ±0.03	2.37 ±0.02	2.36 ±0.03	2.34 ±0.04	2.27 ±0.06	2.33 ±0.08	2.34 ±0.11	2.36 ±0.15	1.98 ±0.07
L-Leucine 5 mM + L-NAME 5 mM	2.49 ±0.03	2.45 ±0.03	2.06 ±0.08	1.89 ±0.07	1.79 ±0.06	1.90 ±0.08	1.89 ±0.07	1.89 ±0.06	1.87 ±0.11	1.68 ±0.05
L-Leucine 5 mM + D-NAME 5 mM	2.47 ±0.02	2.45 ±0.02	2.40 ±0.03	2.20 ±0.05	2.07 ±0.06	1.99 ±0.08	1.85 ±0.07	1.83 ±0.09	1.81 ±0.10	1.92 ±0.10
Tolbutamide 0.185 mM	2.54 ±0.02	2.50 ±0.02	2.47 ±0.03	2.47 ±0.03	2.47 ±0.03	2.17 ±0.06	2.12 ±0.08	2.13 ±0.08	2.17 ±0.09	2.38 ±0.09
Tolbutamide 0.185 mM + L-NAME 5 mM	2.51 ±0.03	2.55 ±0.03	2.34 ±0.05	2.11 ±0.05	2.01 ±0.08	1.60 ±0.06	1.62 ±0.07	1.59 ±0.06	1.56 ±0.05	1.67 ±0.06
Tolbutamide 0.185 mM + D-NAME 5 mM	2.50 ±0.02	2.48 ±0.03	2.42 ±0.03	2.15 ±0.05	2.07 ±0.05	1.63 ±0.06	1.61 ±0.07	1.63 ±0.09	1.58 ±0.06	1.71 ±0.08
KCl 5 mM	2.42 ±0.04	2.37 ±0.02	2.34 ±0.02	2.33 ±0.02	2.30 ±0.03	2.34 ±0.05	2.26 ±0.02	2.22 ±0.07	2.25 ±0.10	1.91 ±0.13
KCl 5 mM + L-NAME 5 mM	2.57 ±.03	2.56 ±0.02	2.11 ±0.03	1.94 ±0.05	1.77 ±0.08	1.85 ±0.11	1.63 ±0.14	1.59 ±0.14	1.48 ±0.12	1.30 ±0.10
KCl 5 mM + D-NAME 5 mM	2.45 ±0.02	2.42 ±0.03	2.40 ±0.05	2.21 ±0.04	2.13 ±0.04	1.96 ±0.06	1.86 ±0.06	1.65 ±0.07	1.66 ±0.09	1.75 ±0.09

Pancreatic effluents are expressed as ml min^{-1}. Values are means ± s.e.mean of 5–7 experiments. Bars show periods of administration of L or D-NAME and of different secretagogues.

medium was a Krebs Ringer Bicarbonate buffer supplemented with 2 g l^{-1} bovine serum albumin (fraction V, Sigma, St-Louis Mo, France) and had the following ionic composition (mM): NaCl 108, KH_2PO_4 1.19, KCl 4.74, $CaCl_2$ 2.54, $MgSO_4$, $7H_2O$ 1.19, $NaHCO_3$ 18. To maintain an adequate oxygen supply and a pH close to 7.35, a mixture of 95% O_2: 5% CO_2 was continuously bubbled through the buffer. Glucose and drugs were added according to the experimental protocols. Circulation of the perfusion medium was performed with a peristaltic pump ensuring a constant output. The perfusion pressure was maintained constant; it was measured with a water manometer, and a pressure limiter allowed part of the medium not accepted by the organ, to return to the origin reservoir. The pressure (35–45 cmH_2O) was selected to obtain a pancreatic flow rate of 2.5 ml min^{-1} measured by collecting samples into graduated test tubes during 1 min; any change in vascular bed resistance induced by drugs could be detected as modifications in pancreatic outflow rate.

Insulin concentrations in pancreatic effluent were determined by a radioimmunological method (Herbert *et al.*, 1965) with Novo rat insulin as standard; the sensitivity of the assay was 0.1 ng ml^{-1}. Insulin outputs were calculated by multiplying the hormone concentration (ng ml^{-1}) in the effluent by the flow rate (ml min^{-1}).

N^{ω}-nitro-L-arginine methyl ester hydrochloride, and N^{ω}-nitro-D-arginine methyl ester hydrochloride, L-arginine hydrochloride, L-leucine, tolbutamide and sodium nitroprusside were from the Sigma Chemical Company.

Our kinetic data are expressed in ng min^{-1} and ml min^{-1} for insulin and effluent outputs respectively; they are given on figures and tables as means ± s.e.mean. In the text, insulin outputs are also given as mean integrated data; for this we calculated the areas under the curves (AUC) for different periods of treatments and values, unless otherwise stated, were divided by the number of minutes. Pancreatic effluents are also referred to as mean integrated values. Both kinetic and integrated data were submitted to analysis of variance followed by the multiple comparison test of Newman-Keuls.

Results

Effect of L- or D-NAME on arginine-induced insulin secretion and pancreatic outflow

Basal insulin levels were low (close to 1 ng min^{-1}) as a result of the low basal glucose concentration (Figure 1). When given alone, L-NAME provoked a progressive slight increase in insulin output (4 ng min^{-1} at the end of treatment). Arginine alone induced a transient (5 min) stimulation of insulin output, which peaked (30.7 ± 2.3 ng min^{-1}) after 2 min. In contrast, when arginine was superimposed on L-NAME, the transient and monophasic pattern of the amino acid effect was strongly potentiated and converted into a marked, sustained biphasic one (100.1 ± 3.9 ng min^{-1} after 2 min). Mean integrated data, corresponding to the 20 min of arginine treatment, averaged 4.1 ± 0.5, 3.8 ± 0.1 and 50.2 ± 3.8 ng min^{-1} for arginine, L-NAME and both drugs when combined, respectively. Unlike L-NAME, D-NAME was ineffective on the 5 mM glucose background (1.44 ± 0.30 ng min^{-1}) and induced a comparatively moderate but significant modification of the arginine-induced second phase, resulting in a mean integrated response

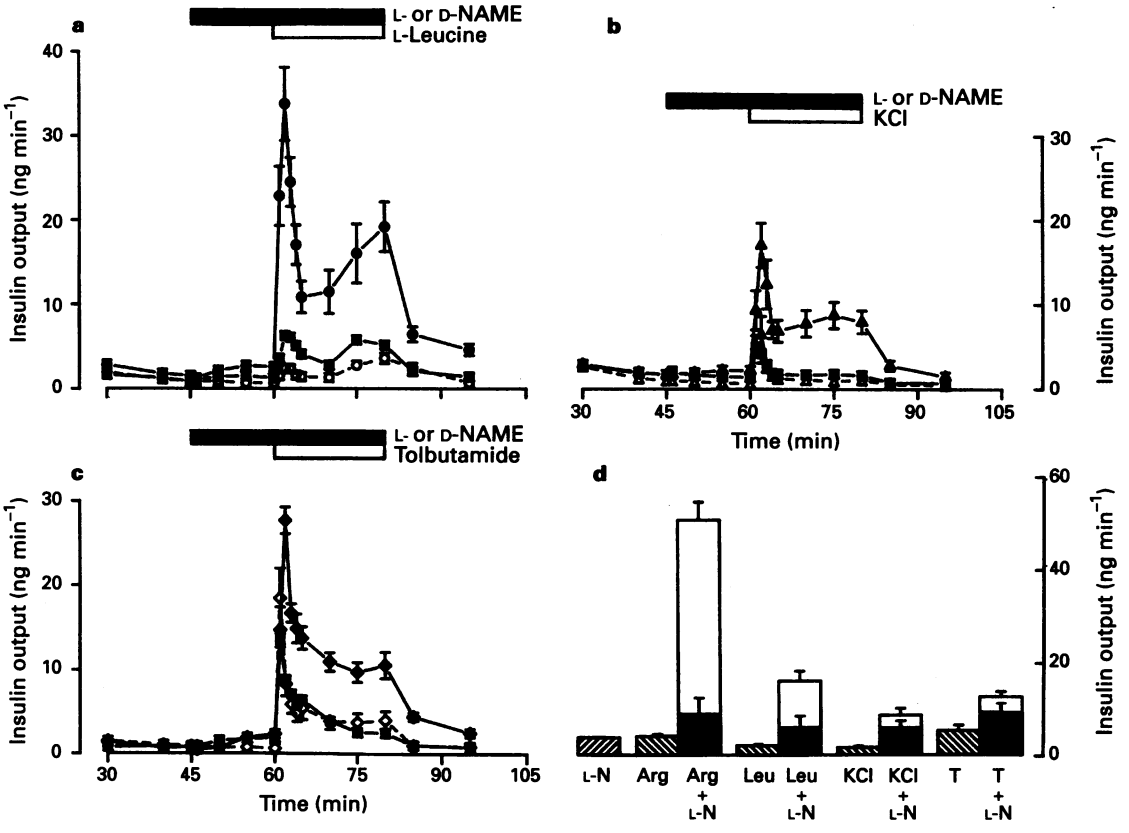

Figure 2 (a,b,c) Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 5 mM) or D-NAME (5 mM) on insulin secretion induced by L-leucine (5 mM), KCl (5 mM) or tolbutamide (0.185 mM) respectively. Points are means ± s.e.mean from 5–7 experiments. (○) Secretagogue alone; (●) secretagogue + L-NAME; (■) secretagogue + D-NAME. The perfusion medium contained glucose (5 mM) throughout. Secretagogues and L- or D-NAME were present for the periods shown by the horizontal bars. (d) Shows the integrated insulin responses from the experiments in Figure 1 and in panels a, b, and c. The filled parts of the open column show the sum of the effects of L-NAME and each secretagogue when added separately. L-N: L-NAME; Arg: Arginine, Leu: L-Leucine; T: Tolbutamide.

of 12.5 ± 1.7 ng min^{-1}. On pancreatic outflow rate (Table 1), L-NAME (5 mM) alone induced a progressive 32% decrease from 2.47 ± 0.02 to 1.67 ± 0.04 ml min^{-1}; arginine alone induced a moderate increase (2.46 ± 0.02 to 2.67 ± 0.05 ml min^{-1}) but was largely unable to reverse the effect of L-NAME (mean integrated outflow rates: 1.90 ± 0.03 and 1.79 ± 0.02 ml min^{-1} in presence and absence of arginine respectively; $P<0.05$). Similar data were obtained for D-NAME (5 mM) (Tables 1 and 2) which decreased mean integrated outflow rate to 1.90 ± 0.03 ml min^{-1}, a value not significantly affected in the presence of arginine (2.01 ± 0.05 ml min^{-1}) (Table 1).

Effect of L-NAME on L-leucine-induced insulin secretion and pancreatic outflow

When L-leucine (5 mM) was added to the 5 mM glucose background the amino-acid induced a small but significant biphasic response (Figure 2a). Mean integrated insulin output for the 20 min of infusion was 2.14 ± 0.22 ng min^{-1}; both phases of leucine effect were significantly increased by about 100% in the presence of D-NAME (4.50 ± 0.31 ng min^{-1}) and strongly potentiated by L-NAME (16.06 ± 2.09 ng min^{-1}). Leucine did not affect basal pancreatic outflow and was unable to reverse the vasoconstrictor effect of L- or D-NAME; however as concerns L-NAME, mean integrated outflow was found slightly less reduced in the presence of the amino acid (1.88 ± 0.07 ml min^{-1}, $P<0.01$ vs 1.63 ± 0.01 with L-NAME alone; see also Table 1).

Effect of L-NAME on KCl and tolbutamide-induced insulin secretion

KCl (5 mM) induced a monophasic release of insulin which peaked at 6.61 ± 1.99 within 2 min (Figure 2b); the mean integrated response reached 1.67 ± 0.24 ng min^{-1} and was increased, in the presence of L-NAME (5 mM), to 8.60 ± 1.47 ng min^{-1}, a value slightly higher than the sum of the effects of both drugs alone (Figure 2b). D-NAME (5 mM) was completely ineffective on KCl-induced insulin secretion. No major observation could be made on pancreatic outflow. Tolbutamide (0.185 mM) provoked a biphasic response of insulin output which peaked at 18.5 ± 3.5 ng min^{-1} in the first min, decreased and then stabilized around 4 ng min^{-1} until the end of administration (Figure 2c). L-NAME (5 mM) alone again slightly increased insulin secretion from 0.8 ± 0.2 to 2.3 ± 0.2 ng min^{-1} ($P<0.01$), but when combined with tolbutamide, a significantly greater response occurred with a pattern that roughly paralleled that obtained with tolbutamide alone. Mean integrated insulin output was 12.6 ± 1.1 ng min^{-1}, a value not far from the sum of mean outputs obtained with tolbutamide (6.7 ± 1.2 ng min^{-1}) and L-NAME alone (3.8 ± 0.1 ng min^{-1}) (Figure 2d) during the corresponding period. In contrast, D-NAME was ineffective on tolbutamide-induced insulin secretion. The sulphonylurea alone provoked a progressive decrease in outflow rate (−12% after 20 min), which enhanced the vasoconstrictor effects of both L and D-NAME (Table 1).

Table 2 Effect of L or D-NAME in presence or absence of SNP on rat pancreatic vascular resistance before and/or during a change in glucose concentration from 5 to 11 mM

	Glucose 5 mM				*Glucose 11 mM or glucose + SNP*					*Glucose 5 mM*
Min	*30*	*45*	*50*	*55*	*60*	*65*	*70*	*75*	*80*	*95*
		L- or D-NAME								
Glucose alone	2.51 ±0.01	2.49 ±0.01	2.49 ±0.02	2.49 ±.03	2.48 ±0.02	2.64 ±0.04	2.63 ±0.05	2.62 ±0.05	2.58 ±0.07	2.32 ±0.03
L-NAME 0.5 mM	2.49 ±0.01	2.46 ±0.02	2.15 ±0.03	2.04 ±0.05	1.96 ±0.06	2.02 ±0.05	1.91 ±0.05	1.90 ±0.04	1.86 ±0.02	1.57 ±0.06
D-NAME 0.5 mM	2.47 ±0.06	2.49 ±0.01	2.45 ±0.08	2.42 ±0.10	2.32 ±0.13	2.18 ±0.09	2.15 ±0.09	2.10 ±0.08	2.05 ±0.09	1.89 ±0.07
L-NAME 5 mM	2.50 ±0.02	2.47 ±0.02	2.16 ±0.02	2.01 ±0.04	1.93 ±0.05	1.91 ±0.06	1.87 ±0.07	1.82 ±0.07	1.82 ±0.07	1.57 ±0.05
D-NAME 5 mM	2.50 ±0.04	2.45 ±0.03	2.03 ±0.04	1.98 ±0.04	1.99 ±0.06	1.95 ±0.07	1.94 ±0.04	1.90 ±0.04	1.87 ±0.06	1.72 ±0.12
L-NAME + SNP 5 mM 30 µM	2.44 ±0.03	2.47 ±0.02	1.93 ±0.10	1.89 ±0.09	1.90 ±0.08	2.97 ±0.07	2.95 ±0.07	2.94 ±0.07	2.94 ±0.06	2.10 ±0.17
SNP 30 µM	2.45 ±0.01	2.45 ±0.01	2.43 ±0.02	2.44 ±0.02	2.44 ±0.01	2.70 ±0.03	2.76 ±0.05	2.80 ±0.04	2.84 ±0.05	2.35 ±0.08
					L-NAME/SNP					
L-NAME 5 mM	2.47 ±0.05	2.49 ±0.03	2.44 ±0.04	2.48 ±0.03	2.46 ±0.03	2.33 ±0.05	2.09 ±0.08	1.92 ±0.10	1.84 ±0.10	1.63 ±0.11
L-NAME + SNP 5 mM 3 µM	2.51 ±0.04	2.48 ±0.02	2.48 ±0.03	2.48 ±0.05	2.54 ±0.05	3.06 ±0.08	3.05 ±0.09	3.07 ±0.10	3.08 ±0.11	1.96 ±0.26
SNP 3 µM	2.48 ±0.07	2.48 ±0.02	2.46 ±0.03	2.48 ±0.03	2.45 ±0.03	2.77 ±0.05	2.78 ±0.06	2.81 ±0.05	2.85 ±0.06	2.40 ±0.06
		L-NAME/SNP								
L-NAME 5 mM	2.46 ±0.03	2.47 ±0.01	2.09 ±0.07	1.94 ±0.06	1.88 ±0.11	1.69 ±0.10	1.71 ±0.08	1.74 ±0.08	1.74 ±0.08	1.49 ±0.06
L-NAME 5 mM + SNP 30 nM	2.46 ±0.01	2.46 ±0.03	2.58 ±0.03	2.56 ±0.03	2.49 ±0.02	1.91 ±0.06	1.81 ±0.06	1.79 ±0.06	1.82 ±0.06	1.61 ±0.06
SNP 30 nM	2.46 ±0.04	2.44 ±0.03	2.53 ±0.03	2.52 ±0.03	2.52 ±0.03	2.49 ±0.04	2.46 ±0.04	2.37 ±0.04	2.38 ±0.02	2.02 ±0.09

Pancreatic effluents are expressed as ml min^{-1}. Values are means ± s.e.mean of 6–8 experiments. Bars show the periods of administration of glucose, L or D-NAME and SNP.

Effect of SNP on L-NAME-induced alterations of insulin response to glucose and vascular flow rate.

Effect of glucose alone The increment in glucose concentration from 5 to 11 mM over the period 60–80 min (Figure 3a) resulted in a clear biphasic response of insulin secretion which peaked (13.5 ± 2.0 ng min^{-1}) after 2 min of high glucose; a minimum was achieved after 5 min (2.7 ± 0.3 ng min^{-1}) and thereafter insulin output rose again to reach 22.9 ± 2.9 ng min^{-1} at the end of infusion. Concerning vascular resistance, glucose induced a dilator effect resulting in a small but significant rise in pancreatic effluent output (Table 2).

Infusion of L-NAME or D-NAME before and during high glucose administration

In the presence of L-NAME, glucose (11 mM) provoked an immediate and sustained rise in insulin output which culminated at 35.1 ± 3.6 ng min^{-1} after 20 min of high glucose (Figure 3a). Mean integrated insulin outputs were 12.7 ± 1.6 and 26.1 ± 2.2 ng min^{-1} ($P<0.01$) respectively in absence and presence of L-NAME during the 20 min of high glucose. In contrast, D-NAME was completely ineffective on glucose-induced insulin secretion both at 0.5 mM (data not shown) and 5 mM. At 0.5 mM L-NAME did not signficantly change mean integrated insulin secretion either during the pretreatment or during the 20 min of high glucose (data not shown). However, a striking observation is that L-NAME at this low concentration blunted the biphasic pattern recorded with glucose alone; indeed, mean integrated insulin secretion corresponding to the first 5 min (6.2 ± 0.8 ng min^{-1}) rose to 9.6 ± 1.0 ng min^{-1} ($P<0.01$) in presence of 0.5 mM L-NAME. Similar data (results not shown) were obtained with 0.1 mM (8.3 ± 0.9 ng min^{-1}, NS) and 0.2 mM (9.8 ± 0.9 ng min^{-1}, $P<0.01$) L-NAME.

Figure 3b shows the effect of SNP (30 μM) on glucose-induced insulin secretion and on L-NAME-induced alteration of insulin response. The NO donor alone did not affect the first phase of β cell response (5.8 ng min^{-1}) but drastically ($P<0.001$) reduced the second one. In the presence of L-NAME, SNP transiently modified the pattern of response recorded with L-NAME alone; 4 and 5 min after the start of high glucose, significantly lower values were obtained (respectively 14.1 ± 1.1 and 11.7 ± 1.3 vs 23.6 ± 1.9 and 22.3 ± 2.1 ng min^{-1} in absence of SNP, $P<0.01$. A similar significant modification of the effect of L-NAME could be observed with SNP (3 μM) (not shown) which alone, unlike at 30 μM, was ineffective on both phases of insulin response to glucose. At 30 nM, SNP did not modify L-NAME induced alteration but in the presence of a high SNP concentration (300 μM; not shown), able alone to decrease the second phase of insulin response to a greater ($P<0.05$) extent than at 30 μM, a clear biphasic and markedly increased insulin response occurred (mean 20 min insulin output reached 36.3 ± 3.9 ng min^{-1} vs 26.1 ± 2.20 in presence of L-NAME alone; $P<0.02$).

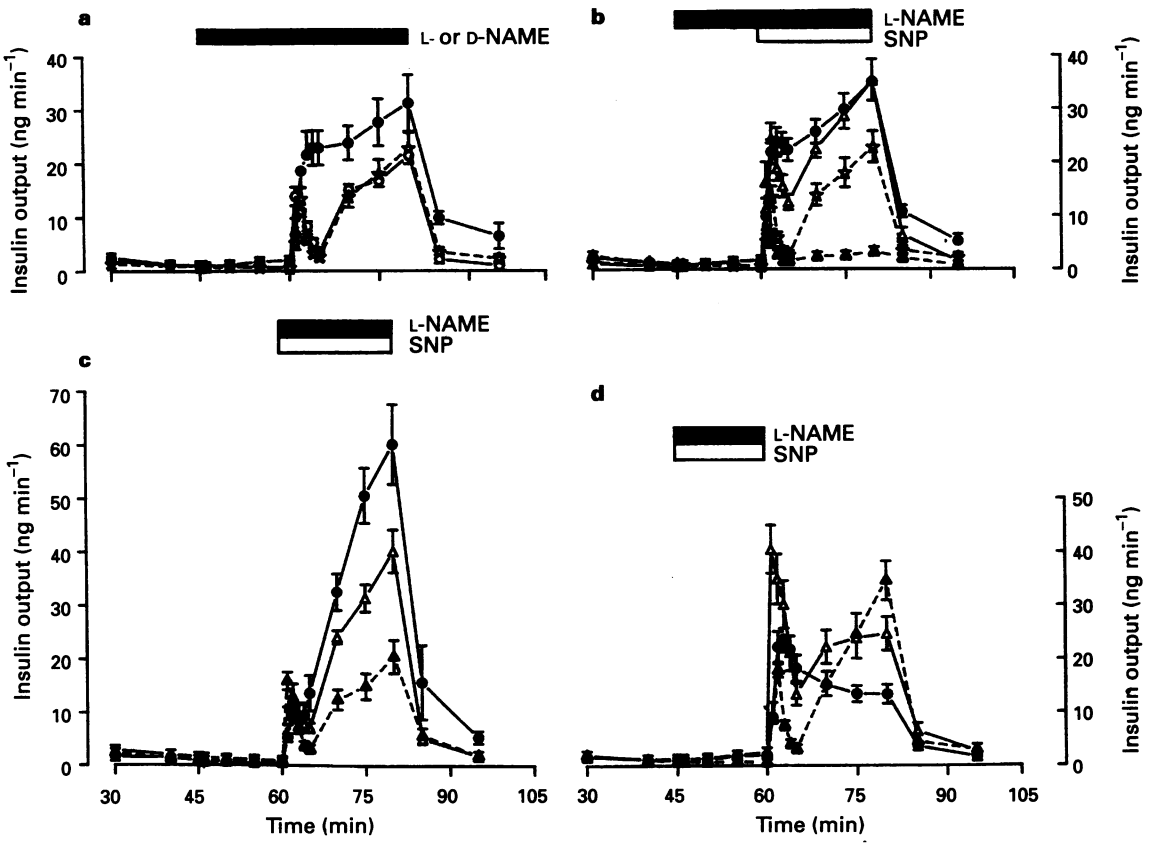

Figure 3 Effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME) or D-NAME (5 mM) or sodium nitroprusside (SNP) on insulin secretion induced by increasing glucose in the perfusion medium from 5 mM to 11 mM for the period 60–80 min. Points are means ± s.e.mean of 6–8 experiments. (a) Increased glucose alone (☆); glucose + L-NAME (●); glucose + D-NAME (○). L- or D-NAME were present for the period 45–80 min as shown by the bar. (b) Increased glucose alone (☆); glucose + L-NAME (●); glucose + L-NAME + SNP (30 μM) (△); glucose + SNP (30 μM) alone (▲). L-NAME or SNP were present for the periods 45–80 min and 60–80 min as shown by the horizontal bars. (c) Glucose and SNP (3 μM) alone (▲); glucose + L-NAME (●); glucose + L-NAME + SNP (△). L-NAME and SNP were present for the period 60–80 min as shown by the horizontal bars. (d) Glucose and SNP (30 nM) alone (▲); glucose + L-NAME (●); glucose + L-NAME + SNP (△). L-NAME and SNP were present for the period 45–60 min as shown by the horizontal bars.

As concerns pancreatic outflow (Table 2) L-NAME at 0.5 and 5 mM provoked a progressive but marked reduction which stabilized after 20 min at about 75% of basal values ($P<0.001$). At 0.5 mM D-NAME induced a delayed and significantly ($P<0.05$) less marked decrease in effluent output than L-NAME but at 5 mM both analogues provoked similar effects. SNP (30 μM) was able to reverse immediately the vasoconstrictor effect of L-NAME and even to induce a dilator effect resulting in a mean integrated outflow significantly greater than in the experiments with glucose alone (58.8 ± 1.3 vs, 52.3 ± 0.8 ml 20 min^{-1}, $P<0.001$). Similar data were obtained with SNP at 300 nM and 3 μM; at 30 nM the NO donor was able to restore pancreatic outflow to basal values.

Infusion of L-NAME during high glucose administration Simultaneous administration of L-NAME (5 mM) and high glucose resulted in a marked modification of the pattern of insulin response: both phases were increased and integrated insulin output for the 20 min of treatment rose to 32.4 ± 3.2 ($P<0.001$) vs 12.7 ± 1.6 ng min^{-1} in absence of the inhibitor (Figure 3c). SNP (3 μM) partly reduced the L-NAME induced alteration. After 5 min of high glucose, insulin output was significantly lower in presence of SNP (7.1 ± 0.9 ng min^{-1}) than with L-NAME alone (13.6 ± 3.3 ng min^{-1}; $P<0.05$), but mean insulin output for the 5 first min remained statistically unchanged. In contrast, the second phase, i.e. mean 15 last min integrated insulin release, was significantly reduced from 43.2 ± 4.3 ng min^{-1} in the absence to 25.2 ± 4.7 in the presence of the NO donor ($P<0.01$).

In this experimental set, L-NAME (5 mM) administered simultaneously with high glucose decreased pancreatic outflow; integrated 20 min outflow reached 42.3 ± 1.3 ml vs 52.3 ± 0.8 ($P<0.001$) in presence of glucose alone. SNP (3 μM) immediately reversed the constrictor effect of L-NAME and induced a significant dilator effect (60.8 ± 1.8 ml 20 min^{-1}, $P<0.001$). It must be noticed that the latter was again significantly more marked than the effect observed when the NO donor was given in the absence of L-NAME ($P<0.02$).

Infusion of L-NAME before high glucose administration L-NAME (5 mM) given 15 min prior to glucose (11 mM) completely changed the kinetics of β cell response (Figure 3d). Insulin output, after an initial peak at 23.5 ± 2.4 ng min^{-1} ($P<0.001$) vs 13.5 ± 2.0 without prior exposure to L-NAME, progressively decayed to values (13.3 ± 1.8 ng min^{-1}) lower than in glucose experiments (23.0 ± 5.6 ng min^{-1}; $P<0.01$). SNP (30 nM), given alone during the 15 min just before the onset of 11 mM glucose, did not significantly affect β cell response; however, in the presence of L-NAME the NO donor restored a biphasic pattern of response but the mean integrated insulin release for the 5 first min was strongly increased. SNP did not modify basal pancreatic outflow (Table 2) but was able to suppress the vasoconstrictor effect of L-NAME during the 15 min pretreatment period; the protective effect of the NO donor disappeared however in the early minutes following cessation of SNP infusion, whereas the effect of L-NAME persisted for more than 30 min after its removal from the perfusate.

Discussion

Our study demonstrates that NO, generated from either exogenous or endogenous arginine sources, is implicated in the regulation of insulin secretion. We provide evidence that this messenger molecule acts as a potent negative modulator of β cell function. Although the effects both of metabolic stimuli and of agents acting primarily via changes in Ca^{2+} fluxes are increased in presence of L-NAME, insulin secretion induced by the former appears to be much more potentiated in presence of the NO synthase inhibitor.

Insertion of a methyl or nitro group onto the guanidino terminus of L-arginine provides potent inhibitors of NO synthase like N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and its methyl ester (L-NAME). We chose to use L-NAME because of its high solubility and because, unlike L-NMMA, it does not interact with the arginine transport system (Bogle *et al.*, 1992) and is not slowly converted into NO and L-citrulline (Klatt *et al.*, 1994). Moreover, L-NAME has been shown to inhibit NO synthase not only *in vitro* in adrenal glands (Palacios *et al.*, 1989), synaptosomes (Knowles *et al.*, 1990) and vascular endothelium, but also *in vivo*, where the inhibitor is able to induce dose-dependent increases in mean systemic arterial blood pressure (Rees *et al.*, 1990). The increase in pancreatic vascular resistance induced by L-NAME, resulting in a substantial reduction of pancreatic outflow already at 100 μM, enables us to extend to the pancreatic vascular bed the important role played by NO formation in the control of basal vascular tone found in different tissues (see Moncada *et al.*, 1991a). It must however be mentioned that D-NAME in the millimolar range is able to induce similar constrictor effects, questioning the specificity of L-NAME-induced vascular effects at high concentration; charge-related depolarizing effects of these two arginine analogues on vascular cells cannot be excluded. Our data with low L-NAME concentrations contrast with those of others (Jansson & Sandler, 1991) who found, *in vitro*, that inhibitors of nitric oxide-forming enzymes do not interfere with the flow distribution within the pancreas and induce no change in perfusion pressure. The reason for such a discrepancy is unknown but might result from differences in experimental conditions. Our vascular bed preparation is very responsive to all vasoactive drugs tested so far, including neurotransmitters and hormones; in order to mimic *in vivo* conditions we used a peristaltic pump ensuring a pulsatile flow which is an important factor triggering a basal dilator tone (Rubanyi *et al.*, 1986). On the same line, it is also of interest in the present study that glucose causes a relaxing effect which is consistent with the report that in rat cultured pancreatic endothelial cells, NO production increases with glucose concentration (Kröncke *et al.*, 1993). Finally, SNP provokes a greater relaxing effect when co-administered with L-NAME than in a non-treated pancreatic vascular bed, which agrees with the demonstration by Moncada *et al.* (1991b) that removal of the basal NO-mediated vasodilator tone leads to a specific supersensitivity to nitrovasodilators both *in vivo* and *in vitro*.

Concerning pancreatic endocrine function, provided that L-NAME is devoid of other major non-specific effects, which is supported by the inability of D-NAME to induce quantitatively similar effects, NO generated by exogenous arginine metabolism is able, in presence of a basal glucose concentration, to reduce strongly the stimulating effect of the amino acid on β cell function. However, since D-NAME provokes a moderate increase in the second phase of arginine-induced insulin secretion, probably due to a charge related effect, the latter might likewise and to the same extent interfere with the blockade of NO synthase by L-NAME and consequently account for a moderate part of the potentiating effect. A similar although less marked potentiation could be observed with L-leucine which is, as its main first metabolic product α-ketoisocaproic acid, able to stimulate insulin secretion (Milner & Hales, 1967; Panten *et al.*, 1972). From the effect of D-NAME on both phases of β cell response to the amino-acid, it can be concluded that a direct depolarizing effect could, again, account for a small part of the potentiation by L-NAME of insulin secretion induced by leucine. This amino acid is not a substrate for NO synthase but α-ketoisocaproate is able within seconds to increase intracellular levels of NADPH and Ca^{2+} (Pralong *et al.*, 1990); both enzymatic co-factors, allowing an increased NO production, might thereby exert an inhibitory feed-back unmasked by L-NAME administration. Such an hypothesis is supported by the relatively low effectiveness of L-NAME in potentiating tolbutamide and KCl-induced insulin secretions.

Concerning glucose, i.e. the β cell physiological stimulus, with respect to the conflicting reports as to the ability of glucose to induce NO formation (Jones *et al.*, 1992; Schmidt *et al.*, 1992) our data agree with an activation of the constitutive form of NO synthase by glucose. However, unlike the stimulating effect on insulin secretion previously proposed, our results bring evidence for an inhibitory effect; addition of L-NAME at increasing concentrations, when performed prior to and during high glucose, progressively converted the biphasic pattern of β cell response into a greater monophasic one, whereas D-NAME was without effect. Related albeit different alterations occurred when blockade was restricted either to basal or to glucose stimulated conditions. In the former, an exaggerated first phase was followed by an abortive second one, which indicates that a basal NO synthase activity is necessary for a normal β cell response to glucose. Furthermore when L-NAME was administered only during high glucose, the second phase of β-cell response was strongly increased, indicating that glucose-induced activation of NO synthase exerts a negative control on insulin response. Taken together these observations suggest that NO produced both as a background in basal conditions and after activation of NO synthase by glucose is implicated, essentially through an inhibitory effect, in the control of the pattern but also the magnitude of β cell response.

As concerns the mechanism by which NO causes inhibition, there is growing evidence that this short-lived oxygen free radical is able to react with a number of naturally occurring sulphydryl containing proteins (Stamler *et al.*, 1992), and functionally essential SH groups in the glucose binding site of β cell glucokinase have been shown to be a target for oxidising agents (Lenzen *et al.*, 1988). In addition, NO also forms iron-nitrosyl complexes with FeS containing enzymes such as aconitase, leading to reversible inactivation of the mitochondrial enzyme (Lancaster & Hibbs, 1990). Such mechanisms have been proposed in studies on the inducible form of NO synthase (Eizirik & Leijerstam, 1994). That L-NAME acts in a great part through an inhibition of NO synthase is supported first by the inability of D-NAME to induce similar effects and second, by our experiments when SNP was used as a palliative treatment. First it must be mentioned that SNP alone induced a sustained inhibition of β cell response to glucose only at high concentrations. Of greater interest are our data obtained with lower SNP concentrations more relevant to physiological conditions. At 3 μM, the NO donor, inactive alone on glucose-induced insulin secretion, reduced partly but continuously the exaggerated second phase provoked by the simultaneous administration of L-NAME and high glucose. Hence L-NAME-induced alteration can, at least partly, result from the blockade of an acute stimulation of NO production. In addition, administration of L-NAME not only during but also before high glucose, shows that basal NO production is also required for a normal control of β cell response; indeed, if SNP partly counteracted L-NAME induced alterations and restored a biphasic response, the sustained inhibitory effect (mentioned above), was converted into a transient one. This suggests that unknown mechanisms related to basal NO synthase activity modulate β cell sensitivity to the inhibitory effect of SNP. Such a modulation might also operate for endogenously produced NO; indeed, when L-NAME administration was restricted to the 15 min before high glucose, simultaneous pretreatment with SNP in the nanomolar range restored a biphasic pattern, but the first phase was found significantly increased. Taken together with the ability of L-NAME in the low millimolar range to blunt the biphasic pattern of insulin secretion, the improvement of L-NAME induced alterations by SNP further stresses the role played by basal and glucose-stimulated NO synthase activity on β cell funtion.

Our study confers to NO a role of physiological relevance when the importance of the biphasic pattern of β cell response to glucose, known to be altered in certain pathological situations related to hyperinsulinism and β cell dysfunction, is considered.

We may conclude that pancreatic endogenous NO production exerts both vascular and endocrine effects. Blockade of its production brings evidence that it acts as an important relaxant factor on the pancreatic vascular bed under basal conditions. As concerns β cell function, NO is implicated in the magnitude of insulin response both to various secretagogues and especially to glucose for which, besides an overall inhibitory tone, NO synthase upon activation could participate in the generation of the biphasic pattern of β cell response.

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