British Journal of Pharmacology (1995) 114, 289-296

Influence of nitric oxide synthase inhibition, nitric oxide and hydroperoxide on insulin release induced by various secretagogues

[1]Georgios Panagiotidis, Björn Åkesson, *Ewa L. Rydell & Ingmar Lundquist

Department of Pharmacology, University of Lund, Lund, Sweden and *Department of Pharmacology, University of Linköping, Linköping, Sweden

1 Recent studies have suggested that the generation of nitric oxide (NO) and hydrogen peroxide (H_2O_2) by islet NO synthase and monoamine oxidase, respectively, may have a regulatory influence on insulin secretory processes. We have investigated the pattern of insulin release from isolated islets of Langerhans in the presence of various pharmacological agents known to perturb the intracellular levels of NO and the oxidation state of SH-groups.

2 The NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) dose-dependently increased L-arginine-induced insulin release. D-Arginine did not influence L-arginine-induced insulin secretion. However, D-NAME which reportedly has no inhibitory action on NO synthase, modestly increased L-arginine-induced insulin release, but was less effective than L-NAME. High concentrations (10 mM) of D-arginine as well as L-NAME and D-NAME could enhance basal insulin release.

3 The intracellular NO donor, hydroxylamine, dose-dependently inhibited insulin secretion induced by L-arginine and L-arginine + L-NAME.

4 Glucose-induced insulin release was increased by NO synthase inhibition (L-NAME) and inhibited by the intracellular NO donor, hydroxylamine. Sydnonimine-1 (SIN-1), an extracellular donor of NO and superoxide, induced a modest suppression of glucose-stimulated insulin release. SIN-1 did not influence insulin secretion induced by L-arginine or the adenylate cyclase activator, forskolin.

5 The intracellular 'hydroperoxide donor' *tert*-butylhydroperoxide in the concentration range of 0.03–3 mM inhibited insulin release stimulated by the nutrient secretagogues glucose and L-arginine. Low concentrations (0.03– 30 μM) of *tert*-butylhydroperoxide, however enhanced insulin secretion induced by the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX).

6 Islet guanosine 3':5'-cyclic monophosphate (cyclic GMP) content was not influenced by 10 mM L-arginine or *tert*-butylhydroperoxide at 3 or 300 μM but was markedly increased (14 fold) by a high hydroxylamine concentration (300 μM). In contrast, islet adenosine 3':5'-cyclic monophosphate (cyclic AMP) content was increased (3 fold) by L-arginine (10 mM) and (2 fold) by *tert*-butylhydroperoxide (300 μM).

7 Our results strongly suggest that NO is a negative modulator of insulin release induced by the nutrient secretagogues L-arginine and glucose. This effect is probably not mediated to any major extent by the guanylate cyclase-cyclic GMP system but may rather be exerted by the S-nitrosylation of critical thiol groups involved in the secretory process. Similarly the inhibitory effect of *tert*-butylhydroperoxide is likely to be elicited through affecting critical thiol groups. The mechanism underlying the secretion-promoting action of *tert*-butylhydroperoxide on IBMX-induced insulin release is probably linked to intracellular Ca^{2+}-perturbations affecting exocytosis.

8 Taken together with previous data the present results suggest that islet production of low physiological levels of free radicals such as NO and H_2O_2 may serve as important modulators of insulin secretory processes.

Keywords: Isolated islets; insulin release; L-arginine; N^G-nitro-D-arginine methyl ester; N^G-nitro-L-arginine methyl ester; D-arginine; hydroxylamine; *tert*-butylhydroperoxide; glucose; cyclic GMP; cyclic AMP

Introduction

Synthesis of nitric oxide (NO) from L-arginine has been identified as an important mechanism implicated in the regulation of cellular function and communication (see Moncada *et al.*, 1991). Very recently, evidence has been obtained for the occurrence of NO synthase activity in both endocrine cells and nerves of the islets of Langerhans (Schmidt *et al.*, 1992; Vincent, 1992; Panagiotidis *et al.*, 1992; Corbett *et al.*, 1993). Previous data (Laychock *et al.*, 1991; Schmidt *et al.*, 1992) suggested that the islet NO-system had a promoting action on insulin secretion. In contrast, however, we recently presented *in vitro* results showing that the NO system in fact is a negative modulator of L-arginine-induced insulin release (Panagiotidis *et al.*, 1992). The insulinotropic action of L-arginine is complex and has partially been attributed to a direct depolarizing effect of the cationic amino acid. Since NO synthase inhibition increased L-arginine-induced insulin release (Panagiotidis *et al.*, 1992) we suggested that the stimulating effect of L-arginine was in part counteracted by the concomitant production of NO. In other tissues the L-arginine-derived NO is thought to act principally by stimulating guanylate cyclase, resulting in the elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Moncada *et al.*, 1991). With regard to insulin secreting mechanisms we recently hypothetised (Panagiotidis *et al.*, 1992) that the inhibitory action of NO might be exerted

[1] Author for correspondence.

through an interaction of NO with thiol groups essential for the secretory process. In fact, it has been known for a long time that certain membrane thiols are involved in the stimulus-secretion coupling of glucose-induced release of insulin (see Hellman *et al.*, 1974; Ammon & Mark, 1985). Thus the formation of S-nitrosothiols may change the balance of critical intracellular and/or membrane thiols of the β-cells. Such a mechanism is in close agreement with our recent proposal that hydrogen peroxide (H_2O_2) generated by the activation of islet monoamine oxidase may inhibit certain insulin secreting processes by oxidizing critical thiol groups (Lundquist *et al.*, 1991; Panagiotidis *et al.*, 1993a,b,c).

The aim of the present investigation was to study in more detail the effect of NO and 'H_2O_2' (as mimicked by *tert*-butylhydroperoxide) on insulin secretion induced by various secretagogues. This was accomplished by means of the NO synthase inhibitor, N^G-nitro-L-arginine methylester (L-NAME), the intracellular NO donor hydroxylamine (De Master *et al.*, 1989), the extracellular donor of NO and superoxide sydnonimine-1 (SIN-1) (Hogg *et al.*, 1992) as well as the intracellular 'hydroperoxide donor' *tert*-butylhydroperoxide (Orrenius & Moldéus, 1984).

Methods

Animals

Female mice of the NMRI strain (B & K, Universal, Sollentuna, Sweden) weighing 25–30 g, were used throughout the experiments. A standard pellet diet (ASTRA-Ewos, Södertälje, Sweden) and tap water were available *ad libitum* before all experiments.

Drugs and chemicals

N^G-nitro-L-arginine methyl ester (L-NAME), D-NAME, L- and D-arginine, 3-isobutyl-1-methylxanthine (IBMX) and hydroxylamine were from Sigma Chemicals, St Louis, MO, U.S.A. *tert*-Butylhydroperoxide was purchased from Merck AG, Darmstadt, Germany. Collagenase (CLS-4) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Bovine serum albumin was from ICN Biomedicals Ltd., High Wycombe, Bucks, U.K. Sydnonimine-1 (SIN-1) was kindly supplied by GEA Ltd., Pharmaceutical Research Laboratories, DK-2650 Hvidovre, Denmark. The radioimmunoassay kits for insulin determination were obtained from NOVO Ltd., Bagsvaerd, Denmark. The antibodies for cyclic GMP and cyclic AMP determination were prepared in our laboratory. The preparation and characterization of the antiserum were made according to Steiner *et al.* (1972). [^{125}I]guanosine or [^{125}I]adenosine 3′:5′-cyclic phosphoric acid was from NEN Dupont, Dreiech, Germany. All other drugs and chemicals were from British Drug Houses Ltd., Poole, Dorset, U.K. or Merck AG., Darmstadt, Germany.

Experimental protocol

Preparation of isolated islets from the mice was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct (Gotoh *et al.*, 1985). Freshly isolated islets were preincubated in a HEPES-supplemented KRB-buffer as previously described (Panagiotidis *et al.*, 1992). After preincubation the buffer was changed and the islets were incubated with the test agents for 60 min at 37°C in an incubation box (30 cycles min^{-1}) if not otherwise stated. The incubation vials contained 8 islets per 1.5 ml buffer except when islets were incubated for cyclic nucleotide determination where 75–80 islets per vial were needed. Immediately after incubation an aliquot of the medium was removed and frozen for subsequent assay of insulin. For analysis of islet cyclic GMP and cyclic AMP the incubation was stopped by the removal of buffer and addition of 0.5 ml ice-cold 10% trichloroacetic acid (TCA), followed by immediate freezing in a −70°C ethanol bath. When assayed, 0.5 ml H_2O was added and the samples were sonicated for 3 × 5 s in a Branson Sonifier Cell disrupter followed by centrifugation at 1.100 *g* for 15 min. The supernatants were collected and extracted with 4 × 2 ml of water-saturated diethyl ether. The aqueous phase was recovered and freeze dried, using a Lyovac GT 2 freeze dryer. The residue was then dissolved in 450 µl 50 mM Na-acetate buffer (pH 6.2). Both cyclic GMP and cyclic AMP were determined by radioimmunoassay according to Steiner *et al.* (1972). In order to improve sensitivity of the assays, the samples were acetylated with acetic acid anhydride as described by Harper & Brooker (1975).

Results

Effect of NO synthase inhibition on basal and stimulated insulin secretion

Figure 1a shows the effect of different concentrations of the NO synthase inhibitor L-NAME (an L-arginine analogue) on basal insulin secretion (7 mM glucose) from isolated islets. L-NAME-concentrations of 0.1–5.0 mM did not significantly influence basal insulin release. A high concentration of L-NAME (10 mM), however, induced a 3 fold increase in insulin secretion.

Figure 1b and 1c illustrate the influence of NO synthase inhibition on insulin release stimulated by L-arginine and glucose, respectively. Concentrations of L-NAME which did not affect basal insulin secretion (Figure 1a) dose-dependently potentiated L-arginine-induced insulin release (Figure 1b). Furthermore, NO synthase inhibition did also increase glucose-stimulated insulin secretion (Figure 1c).

Effect of D-arginine and D-NAME on basal and L-arginine-stimulated insulin secretion

Figure 2a compares the effects of L-arginine and D-arginine, the stereoisomer of L-arginine, on insulin release. Similar to 5 mM L-NAME (Figure 1a) 5 mM D-arginine had no insulin releasing effect (Figure 2a). However, unlike L-NAME, D-arginine did not influence L-arginine-induced insulin release. Figure 2b shows that D-NAME, which similar to D-arginine, reportedly is devoid of NO synthase inhibitory properties modestly increased L-arginine-induced insulin release but to a significantly lower level than did L-NAME. A high concentration of D-arginine (10 mM) (Figure 2a), however, induced a moderate insulin response which was approximately 4 times less than that of 10 mM L-arginine (Figure 2b). D-NAME (10 mM) could stimulate basal insulin release to the same extent as L-NAME (Figure 2b).

Effect of the intracellular NO donor, hydroxylamine, on the secretion of insulin induced by L-arginine and glucose

Figure 3 illustrates the effect of the intracellular NO donor hydroxylamine on insulin secretion induced by 10 mM L-arginine in the presence of 7 mM basal glucose (Figure 3a). Figure 3b shows the effect of hydroxylamine on insulin release stimulated by 16.7 mM glucose. The NO donor dose-dependently inhibited L-arginine stimulated insulin release which was almost totally suppressed at 3 mM hydroxylamine. Glucose-induced insulin release appeared less sensitive to the drug, although a total suppression was again achieved at 3 mM hydroxylamine. Hydroxylamine did not influence basal (7 mM glucose) insulin secretion (data not shown).

Effect of the extracellular NO and superoxide donor SIN-1 on insulin secretion induced by glucose, L-arginine and forskolin

The next series of experiments examined the effect of the NO and superoxide donor SIN-1, which is believed to release NO

and superoxide extracellularly. Figure 4a shows that SIN-1 significantly reduced insulin release stimulated by 16.7 mM glucose. This inhibition was modest and detectable already at 0.3 μM SIN-1 but was less pronounced than the inhibitory effect of hydroxylamine (Figure 3b). SIN-1 had no influence on insulin secretion induced by L-arginine (Figure 4b) or by the adenylate cyclase activator, forskolin (Figure 4c). Note that the SIN-1 experiments were run only for 35 min because of the reported rapid deterioration of this compound.

Effect of the intracellular 'hydroperoxide donor' tert-butylhydroperoxide on insulin secretion induced by L-arginine, glucose and isobutylmethylxanthine (IBMX)

In analogy with the experiments on intracellular delivery of NO by hydroxylamine we then examined the effect of intracellular hydroperoxide by employing *tert*-butylhydroperoxide. Data from previous *in vivo* experiments suggested that an intracellular generation of H_2O_2 by the activation of islet monoamine oxidase might impair glucose-induced but promote IBMX-induced insulin release (Lundquist *et al.*, 1991; Panagiotidis *et al.*, 1993b,c, 1994). Figure 5a and b illustrate the effect of *tert*-butylhydroperoxide on insulin secretion induced by 10 mM L-arginine and 16.7 mM glucose, respectively. The 'hydroperoxide donor' markedly suppressed both L-arginine- and glucose-induced insulin release. In contrast insulin secretion induced by IBMX, an activator of the cyclic AMP system, was enhanced by low levels of *tert*-butylhydroperoxide (0.03–30 μM) (Figure 5c). A very high concentration of *tert*-butylhydroperoxide (3 mM) suppressed IBMX-induced insulin release. *tert*-Butylhydroperoxide did not influence basal (4 mM glucose) insulin secretion (data not shown).

Comparative effects of hydroxylamine and tert-butylhydroperoxide on insulin secretion induced by L-arginine + L-NAME

In order to examine the effect of hydroxylamine and *tert*-butylhydroperoxide on L-arginine-induced insulin secretion in the absence of endogenous NO production, a series of experiments was performed with the NO synthase inhibitor L-NAME. Figure 6a shows that hydroxylamine dose-dependently suppressed L-arginine-induced insulin release in the presence of L-NAME with a total inhibition at 3 mM. Figure 6b shows that *tert*-butylhydroperoxide was less potent in this respect displaying an inhibitory action of approximately 50%

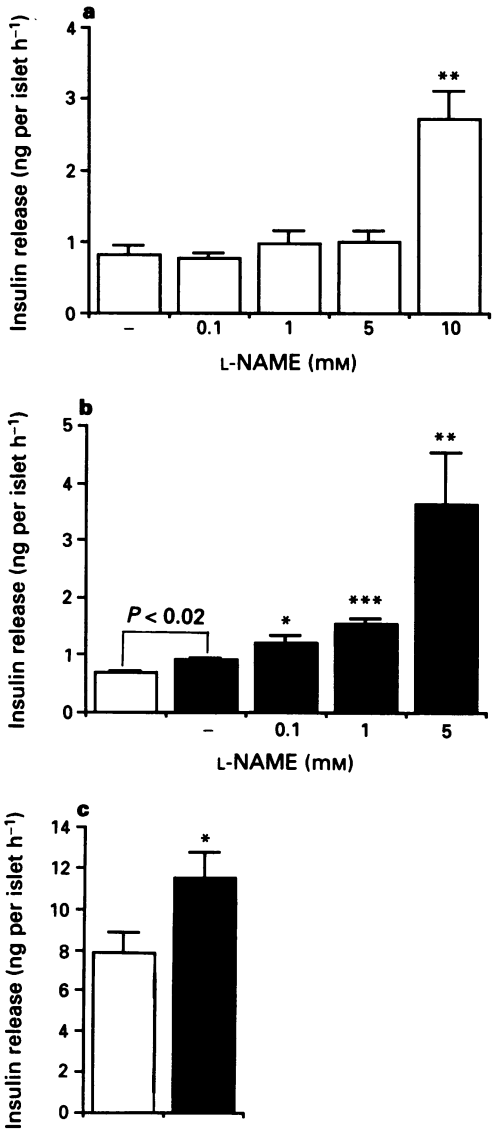

Figure 1 (a) Effect of different concentrations of N^G-nitro-L-arginine methyl ester (L-NAME) on basal insulin secretion (7 mM glucose) from isolated islets. Values are mean ± s.e.mean from 6–10 batches of islets $^{**}P<0.01$ *vs* basal control. (b) Influence of different concentrations of L-NAME on insulin release from isolated islets stimulated by L-arginine 5 mM in the presence of basal glucose 7 mM (solid columns). Glucose 7 mM basal control is denoted by an open column. Values are mean ± s.e.mean from 6–10 batches of islets, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to 5 mM L-arginine without addition of L-NAME. (c) Glucose-induced (16.7 mM) insulin release from isolated islets in the absence (open column) or presence (solid column) of 5 mM L-NAME. Values are mean ± s.e.mean from 6–10 batches of islets, $^{*}P<0.05$.

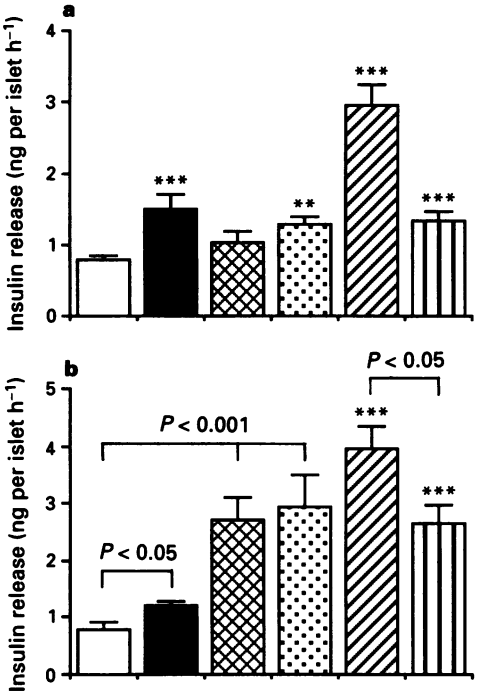

Figure 2 (a) Effect of L-arginine 5 mM (solid column), D-arginine 5 mM (cross-hatched column), L-arginine 5 mM + D-arginine 5 mM (stippled column), L-arginine 10 mM (hatched column) and D-arginine 10 mM (striped column) on insulin secretion from isolated islets in the presence of 7 mM glucose. Basal control (7 mM glucose) is denoted by an open column. Values are mean ± s.e.mean from 6–10 batches of islets, $^{**}P<0.01$, $^{***}P<0.001$ compared to basal control. (b) Effect of L-arginine 5 mM (solid column), L-NAME 10 mM (cross-hatched column), D-NAME 10 mM (stippled column), L-arginine 5 mM + L-NAME 5 mM (hatched column) and L-arginine 5 mM + D-NAME 5 mM (striped column) on insulin secretion from isolated islets in the presence of 7 mM glucose. Basal control (7 mM glucose) is denoted by an open column. Values are mean ± s.e.mean from 6–10 batches of islets, $^{***}P<0.001$, compared to L-arginine 5 mM.

of the L-arginine + L-NAME insulin response at 0.3 and 3 mM.

Effect of L-arginine, hydroxylamine and tert-butylhydroperoxide on islet cyclic GMP and cyclic AMP levels

Table 1 shows the effect of 10 mM L-arginine as well as concentrations of 3 and 300 μM hydroxylamine and *tert*-butylhydroperoxide on the levels of cyclic GMP and cyclic AMP in isolated mouse islets at 7 mM glucose. The glucose concentration in the control incubation medium was 7 mM. It is seen that only 300 μM hydroxylamine influenced the cyclic GMP levels. At this concentration the NO donor evoked a 14 fold increase in islet cyclic GMP compared to control levels. In contrast, the cyclic AMP levels were increased by L-arginine as well as by the 300 μM concentration of *tert*-butyl-hydroperoxide (Table 1).

Discussion

The present results show that the L-arginine-analogues L-NAME (an NO synthase inhibitor), and D-arginine (which does not generate NO), acted differently on insulin release when incubated together with L-arginine. Increasing concentrations of L-NAME (0.1–5.0 mM), which did not influence basal insulin release, induced a dose-related potentiation of the insulin response evoked by a submaximal concentration of L-arginine (5 mM). D-Arginine (5 mM), however, did not influence L-arginine-induced insulin release. On the other hand D-NAME (5 mM) which reportedly is devoid of NO synthase-inhibitory properties (Radomski *et al.*, 1990; Rees *et al.*, 1990) also increased the insulin release induced by L-arginine but was less effective than L-NAME. Moreover, a high concentration (10 mM) of L-NAME, D-NAME or D-arginine could *per se* induce insulin secretion, D-arginine being less efficient in this respect. The data suggest that the observed effects of the L-arginine analogue L-NAME, at least partially, could be ascribed to its cationic properties. However, the fact the D-NAME was less effective than L-NAME in increasing L-arginine-induced insulin release strongly suggests that part of the effect was due to NO synthase inhibi-

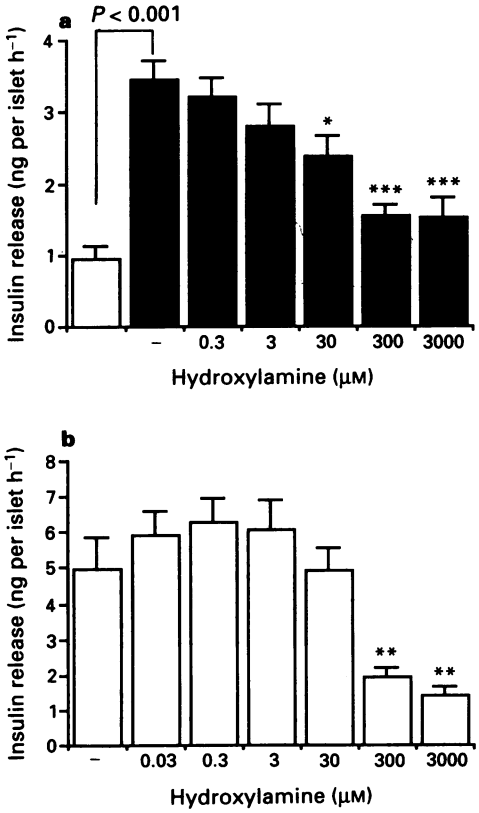

Figure 3 (a) Effect of different concentrations of hydroxylamine on insulin release from isolated islets stimulated by L-arginine 10 mM in the presence of basal glucose 7 mM (solid columns). Glucose 7 mM basal control is also shown (open column). Values are mean ± s.e.mean from 9 batches of islets, $*P<0.05$, $***P<0.001$ compared to 10 mM L-arginine without addition of hydroxylamine. (b) Effect of different concentrations of hydroxylamine on glucose-induced insulin secretion (16.7 mM glucose) from isolated islets. Values are mean ± s.e.mean from 9 batches of islets, $**P<0.01$.

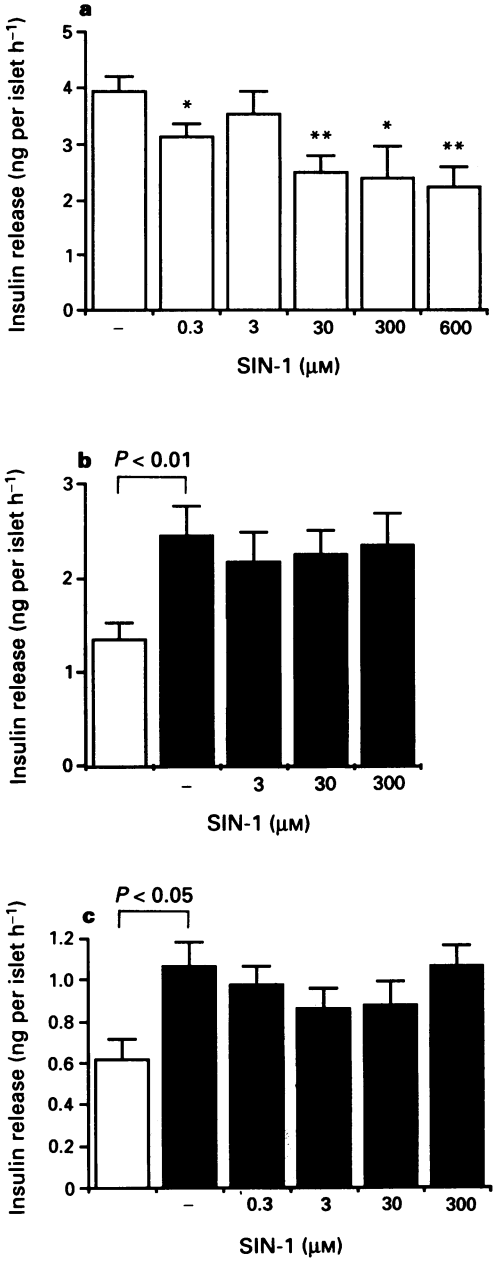

Figure 4 (a) Effect of different concentrations of sydnonimine-1 (SIN-1) on glucose induced insulin secretion (16.7 mM glucose) from isolated islets during a 35 min incubation period. Values are mean ± s.e.mean from 9 batches of islets, $*P<0.05$, $**P<0.01$ compared to 16.7 mM glucose without addition of SIN-1. (b) Effect of SIN-1 on L-arginine-stimulated (10 mM) insulin release in the presence of 7 mM glucose (solid columns). Glucose 7 mM basal control is also shown (open column). Values are mean ± s.e.mean from 7 batches of islets. (c) Effect of SIN-1 on insulin release from isolated islets stimulated by forskolin (20 μM) in the presence of 4 mM glucose (solid columns). Glucose 4 mM basal control is also shown (open column). Values are mean ± s.e.mean from 9 batches of islets.

tion. It should be noted, however, that the stereoisomer D-arginine, which carries the same cationic properties as L-arginine and does not generate NO, was a weaker insulin secretagogue than L-arginine even in the presence of L-NAME. This suggests that other unknown factors may contribute to the difference in the insulin releasing action of D- and L-arginine. One explanation might be the existence of different transport mechanisms across the plasma membrane as previously shown in endothelial cells (Bogle *et al.*, 1992). Thus, D-arginine might not be accumulated as rapid as or to the same extent as L-arginine and L- or D-NAME. However, taken together our data indicate that L-arginine has a dual

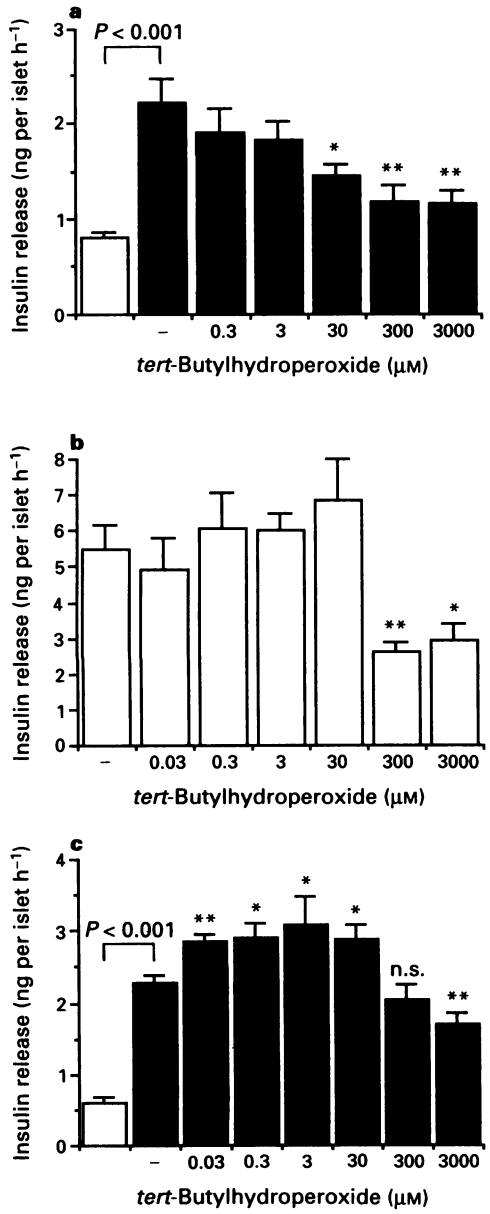

Figure 5 (a) Effect of different concentrations of *tert*-butylhydroperoxide on insulin release from isolated islets stimulated by L-arginine 10 mM in the presence of basal glucose 7 mM (solid columns). Glucose 7 mM basal control is also shown (open column). Values are mean ± s.e.mean from 6–9 batches of islets, $*P<0.05$, $**P<0.01$ compared to 10 mM L-arginine without addition of *tert*-butylhydroperoxide. (b) Effect of different concentrations of *tert*-butylhydroperoxide on glucose-induced insulin secretion (16.7 mM glucose) from isolated islets. Values are mean ± s.e.mean from 6 batches of islets, $*P<0.05$, $**P<0.01$ compared to 16.7 mM glucose without addition of *tert*-butylhydroperoxide. (c) Effect of different concentrations of *tert*-butylhydroperoxide on insulin release from isolated islets stimulated by isobutylmethylxanthine (IBMX) 1 mM in the presence of basal glucose 4 mM (solid columns). Glucose 4 mM basal control is also shown (open column). Values are mean ± s.e.mean from 9 batches of islets, $*P<0.05$, $**P<0.01$ compared to 1 mM IBMX without addition of *tert*-butylhydroperoxide.

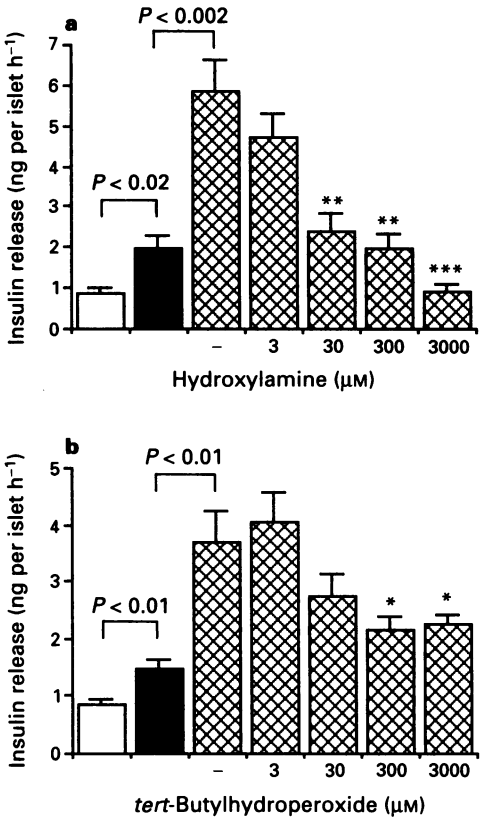

Figure 6 (a) Influence of different concentrations of hydroxylamine on insulin release from isolated islets stimulated by L-arginine 5 mM + N^G-nitro-L-arginine methyl ester (L-NAME) 5 mM in the presence of basal glucose 7 mM (cross-hatched columns). Effects of basal glucose (7 mM) (open column), and L-arginine 5 mM (solid column) are also shown. Values are mean ± s.e.mean from 6–9 batches of islets, $**P<0.01$, $***P<0.001$ compared to 5 mM L-arginine + L-NAME without addition of hydroxylamine. (b) Influence of different concentrations of *tert*-butylhydroperoxide on insulin release from isolated islets stimulated by L-arginine 5 mM + L-NAME 5 mM in the presence of basal glucose 7 mM (cross-hatched columns). Effects of basal glucose (7 mM) (open column), and L-arginine 5 mM (solid column) are also shown. Values are mean ± s.e.mean from 6–9 batches of islets, $*P<0.05$ compared to 5 mM L-arginine + L-NAME 5 mM without addition of *tert*-butylhydroperoxide.

Table 1 Accumulated levels of cyclic GMP and cyclic AMP in isolated islets after a 60 min incubation period in the presence of 10 mM L-arginine or different concentrations of hydroxylamine and *tert*-butylhydroperoxide

	Cyclic GMP (amol/islet)	*Cyclic AMP* (fmol/islet)
Control	333 ± 14	6.19 ± 1.27
L-Arginine 10 mM	358 ± 39	17.12 ± 3.12**
Hydroxylamine 3 μM	351 ± 19	9.66 ± 1.25
Hydroxylamine 300 μM	4675 ± 950***	7.88 ± 1.26
tert-Butylhydroperoxide 3 μM	481 ± 74	5.94 ± 0.62
tert-Butylhydroperoxide 300 μM	320 ± 41	11.20 ± 0.71*

Basal medium (control) contained 7 mM glucose + 0.2 mM IBMX.
Means ± s.e.mean from 5–7 batches of islets. Asterisks (*) denote significant difference *vs* control. $*P<0.02$; $**P<0.005$; $***P<0.001$.

action on insulin release as previously suggested (Panagiotidis *et al.*, 1992). Thus the cationic depolarizing action of L-arginine promotes insulin secretion, whereas the NO derived from L-arginine metabolism negatively modulates the secretory process.

The results from previous investigations with rat islets or cells from the clonal pancreatic β-cell line, HIT-T15, as well as RINm5F insulinoma cells using the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (Laychock *et al.*, 1991; Schmidt *et al.*, 1992) suggested that NO enhances L-arginine-stimulated insulin release. The reason for this discrepancy in results is presently unclear. However, the β-cell lines may have a different reaction pattern compared with normal islets. Further, we recently observed that during our incubation conditions L-arginine-induced insulin release also from rat islets was enhanced by NO synthase inhibition (data not shown). These results are also compatible with previous data on the effect of L-NMMA on insulin release from cultured rat islets (Jansson & Sandler, 1991). Moreover, in contrast to previous reports (Laychock *et al.*, 1991; Schmidt *et al.*, 1992; Jones *et al.*, 1992) our present results suggest that glucose-induced insulin secretion (16,7 mM glucose) is increased in the presence of NO synthase inhibition. This may suggest that L-NAME inhibited a basal 'tone' of NO generation within the isolated islets in the presence of high glucose and/or that L-NAME exerted a cationic potentiation of glucose-induced insulin secretion. The fact that 10 mM of either sterioisomeric form of NAME alone released the same amounts of insulin favours the latter assumption. Thus, to study in more detail the inhibition of NO synthase activity in islet β-cells, a new generation of NO synthase inhibitors has to be developed that hopefully does not share the cationic properties of the L-arginine analogues.

In accordance with our suggestion that NO in fact is a negative modulator of L-arginine-induced insulin secretion, the intracellular NO donor, hydroxylamine, displayed a dose-dependent inhibition of the insulin response to L-arginine alone and to the combined action of L-NAME + L-arginine. Moreover, glucose-induced insulin secretion was also markedly inhibited by intracellular NO generation induced by hydroxylamine.

Hydroxylamine is a known precursor of NO in biological systems and it has even been suggested that it may be an intermediate in the conversion of L-arginine to NO (DeMaster *et al.*, 1989). Further, previous data have suggested that hydroxylamine easily penetrates into cells where it is oxidized to NO and exerts its effects. Catalase is known to catalyze this reaction (DeMaster *et al.*, 1989).

With regard to SIN-1 the effect is probably in the interstitial environment as, from its chemical structure, this compound is not likely to gain access to the cell interior. Hence the 'mixed' free radicals, NO and superoxide, generated by SIN-1 (Hogg *et al.*, 1992) may act mainly extracellularly. It has previously been shown that direct oxidation of β-cell superficial thiols lead to Ca^{2+} uptake and subsequent increase of insulin secretion at low glucose (Ammon & Mark, 1985). However, oxidation of intracellular thiols inhibits glucose-induced insulin secretion (Ammon & Mark, 1985). Thus the action of SIN-1 is very complex, since this compound generates both superoxide and NO, which then can act both extra- and intracellularly. Apparently the intracellular effect of the evolved NO and superoxide dominates with regard to glucose-induced insulin release, whereas insulin release stimulated by L-arginine is unaffected (balance between extracellular and intracellular effects of free radicals?). Similarly forskolin-induced insulin release was unaffected by SIN-1 even at a very low glucose concentration (cyclic AMP is known to lower the threshold for glucose-induced insulin release) confirming earlier data showing that activation of the cyclic AMP system by (−)-isoprenaline is not affected by thiol oxidation (Ammon & Mark, 1985). Therefore, it should be noted that although the main effect of NO has been ascribed to the intracellular activation of guanylate cyclase, recent observations have suggested that NO might exert its action also through cyclic GMP-independent events (Stamler *et al.*, 1992). Thus S-nitrosylation of critical thiol groups on e.g. enzyme and receptor proteins has been suggested as a potential mechanism for modification of proteins with important regulatory functions (Stamler *et al.*, 1992).

The combined data on the effects of L-NAME on one hand and the intracellular NO donor hydroxylamine on the other thus strongly suggest that NO is a negative modulator of insulin secretion induced by L-arginine and glucose. However, it should be noted that up to date biochemical evidence for the presence of a constitutive NO synthase in islet tissue is restricted to the report of Schmidt *et al.* (1992) who used HIT T15 clonal pancreatic β-cells. Measurement of nitrite production as an indicator of NO synthase activity has been used by several authors (cf. Moncada *et al.*, 1991; Corbett *et al.*, 1993). However, this may be of limited value in short-term incubation of islet tissue, since according to our hypothesis much of the NO produced is 'trapped' by S-nitrosylation, (see also Stamler *et al.*, 1992). Moreover, comparatively high concentrations of NO synthase inhibitors (in the order of 10^{-4}–10^{-3} M) have been used to detect an inhibitory action on nitrite production from cultured islets (Welsh *et al.*, 1991; Corbett *et al.*, 1993). Therefore, a more detailed biochemical characterization of islet NO synthase(s) is needed. On the other hand, the immunohistochemical evidence for the occurrence of a constitutive NO synthase in islet β-cells seems to be very strong, since this has been demonstrated with three different antisera (Schmidt *et al.*, 1992; Corbett *et al.*, 1993). Moreover, islets cultured with 0.5 mM L-NMMA displayed a substantially reduced NADPH diaphorase staining (Corbett *et al.*, 1993). With regard to the inhibition of NO synthase it could be argued, however, that we used a comparatively high concentration of L-NAME (5 mM) (Moncada *et al.*, 1991; Förstermann *et al.*, 1991) in the L-arginine *vs.* L-NAME interaction experiments. Since the inhibiting action of L-NAME is competitive (Moncada *et al.*, 1991) and since concentrations of 5–10 mM L-arginine are needed to obtain a reliable insulin release (Gerich *et al.*, 1974) it was necessary to use the highest concentration of L-NAME that did not *per se* affect insulin release (cf. Figure 1a).

It has been known for a long time (see Hellman *et al.*, 1974; Ammon & Mark, 1985) that superficial thiols are of importance for certain insulin secretagogues, and that the reduced state of the intracellular GSH/GSSG system, which maintains the superficial thiols in the reduced state, is a prerequisite for glucose-stimulated Ca^{2+} uptake and insulin release. Hence it is not inconceivable that NO-induced S-nitrosylation may impair the function of critical thiol groups of importance for insulin secretion induced by glucose and L-arginine. This might be accomplished either directly or as a consequence of deranging the balance of the GSH/GSSG system. Our present finding that L-arginine-induced insulin release is more sensitive to NO-inhibition than insulin secretion stimulated by glucose is in good agreement with previous observations (Ammon & Mark, 1985) showing that glucose metabolism increases the GSH/GSSG ratio by generating NADPH from the pentose shunt.

Similar to the described effects of NO production, it has been proposed that the H_2O_2 evolution induced by the activation of islet monoamine oxidase activity negatively modulates glucose-induced insulin secretion by impairing the function of critical thiol groups (Lundquist *et al.*, 1991; Panagiotidis *et al.*, 1993a,b,c). The present results, which show that the intracellular 'hydroperoxide donor' *tert*-butylhydroperoxide inhibited insulin secretion induced by glucose and L-arginine strongly support such an assumption. In fact the pattern of insulin secretory suppression induced by *tert*-butylhydroperoxide ('H_2O_2') and hydroxylamine (NO) was remarkably similar, although on a molar basis NO appeared more potent in this respect. It should be emphasized, however, that although both hydroxylamine and *tert*-butylhydroperoxide

are believed to exert their effects intracellularly, the physiological activation of NO synthase (NO) and monoamine oxidase (H_2O_2) may induce a much more localized increase of these messengers, which makes precise comparisons difficult. Moreover, it is not finally established that *tert*-butylhydroperoxide has exclusively intracellular actions (Orrenius & Moldéus, 1984).

Recent *in vivo* studies have suggested that activation of islet monoamine oxidase by certain cyclic AMP-increasing agents such as the β_2-adrenoceptor agonist, terbutaline (Panagiotidis *et al.*, 1993b,c) and the phosphodiesterase inhibitor IBMX (Panagiotidis *et al.*, 1994) could facilitate the insulin releasing action of these agents by degrading inhibitory monoamines and producing small levels of exocytosis-promoting H_2O_2. In contrast to insulin release stimulated by nutrients such as glucose and L-arginine, the secretory process induced by cyclic AMP activating agents seem to operate largely independently of the GSH/GSSG balance as well as of extracellular Ca^{2+} (Ammon & Mark, 1985; Draznin, 1988; Salehi & Lundquist, 1993). The present *in vitro* results, which show that very small levels (0.03–30 μM) of the intracellular 'hydroperoxide donor' *tert*-butylhydroperoxide indeed enhanced IBMX-induced insulin release from isolated islets are in good agreement with our hypothesis. The detailed mechanism of action of this facilitation of IBMX-induced insulin release by hydroperoxide remains to be elucidated. However, it is known from other tissues that peroxides can promote granule fusion with membranes, and that H_2O_2 production can result in perturbation of intracellular Ca^{2+} homeostasis leading to an increase in cytosolic free Ca^{2+} concentration (Creutz, 1981; Orrenius & Moldeús, 1984).

Finally, a concentration of L-arginine (10 mM) which induced a large stimulation of insulin release in the presence of a physiological concentration of glucose (7 mM) did not affect islet cyclic GMP levels (Table 1). These results are in accordance with a recent study (Jones *et al.*, 1992) and do not suggest that L-arginine-derived NO initiates insulin secretion through increasing islet cyclic GMP content as previously proposed (Laychock *et al.*, 1991). In fact, the NO donor, hydroxylamine (at a concentration of 300 μM) induced a marked increase in islet cyclic GMP levels. This concentration of hydroxylamine almost totally *suppressed* insulin secretion induced by glucose or L-arginine. *tert*-Butylhydroperoxide on the other hand did not significantly influence the islet cyclic GMP content. Hence, most evidence thus far suggests that NO is a negative modulator of glucose- and L-arginine-induced insulin release and that cyclic GMP (at least at very high levels) may even contribute to this suppressive action (see also Vara & Tamarit-Rodriguez, 1991).

With regard to islet cyclic AMP we were able to show that 10 mM L-arginine brought about an increase of this nucleotide in islet tissue. This novel finding suggests that raised cyclic AMP levels may contribute to the insulin releasing action of L-arginine. However, it cannot be excluded that effects on cyclic AMP in the glucagon cells may be involved. Similarly, the stimulating effect of *tert*-butylhydroperoxide (300 μM) on islet cyclic AMP levels does not correlate to insulin release and thus may reflect either cyclic AMP changes in the glucagon cells or simply illustrate the notion that cyclic AMP itself is not an initiator of insulin release (Ammon & Mark, 1985).

In conclusion, suppression and enhancement of NO generation by the NO synthase inhibitor, L-NAME, on the one hand, and the intracellular NO donor hydroxylamine on the other, induced paralell changes in insulin release stimulated by L-arginine and glucose. These findings are compatible with NO being a negative modulator of the secretory events. The main effect of NO is probably not exerted through elevated cyclic GMP levels but rather through S-nitrosylation of certain thiol groups critical for the secretory process. Such an assumption is strengthened by the observation that the intracellular 'hydroperoxide donor' *tert*-butylhydroperoxide, a recognized thiol oxidant, induced almost similar changes in the insulin releasing pattern. Cyclic AMP activating secretagogues such as forskolin and IBMX appeared much less sensitive to the inhibitory action of NO and hydroperoxide. In contrast, small levels of hydroperoxide were found to enhance IBMX-induced insulin release. Thus the present data showing a differential *in vitro* effect of *tert*-butylhydroperoxide on insulin secretion induced by glucose and IBMX, respectively, are in good agreement with the results of previous *in vivo* experiments (Lundquist *et al.*, 1991; Panagiotidis *et al.*, 1993a,b,c) suggesting that the H_2O_2 generated by activation of islet monoamine oxidase activity inhibits glucose-induced insulin secretion and promotes insulin release stimulated by IBMX. Hence, taken together with previous data the present results suggest that islet production of low physiological levels of free radicals such as NO and H_2O_2 may serve as important modulators of insulin secretory processes.

This study was supported by the Swedish Medical Research Council (14X-4286), the Swedish Diabetes Association, the Crafoord Foundation, The Novo Nordisk Foundation and the Medical Faculty, University of Lund, Sweden. The technical assistance of Elsy Ling and Britt-Marie Nilsson is gratefully acknowledged.

References

AMMON, H.P.T. & MARK, M. (1985). Thiols and pancreatic β-cell function: a review. *Cell. Biochem. Funct.*, **3**, 157–171.

BOGLE, R.G., MONCADA, S., PEARSON, J.D. & MANN, G.E. (1992). Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br. J. Pharmacol.*, **105**, 768–770.

CORBETT, J.A., WANG, J.L., MISKO, T.P., ZHAO, W., HICKEY, W.F. & MCDANIAL, M.L. (1993). Nitric oxide mediates IL-1β-Induced islet dysfunction and destruction: Prevention by dexamethasone. *Autoimmunity*, **15**, 145–153.

CREUTZ, C.E. (1981). cis-Unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. *J. Cell. Biol.*, **91**, 247–256.

DEMASTER, E.G., RAU, L., ARCHER, S.L. & WEIR, E.K. (1989). Hydroxylamine is a vasorelaxant and a possible intermediate in the oxidative conversion of L-arginine to nitric oxide. *Biochem. Biophys. Res. Commun.*, **163**, 527–533.

DRAZNIN, B. (1988). Intracellular calcium, insulin secretion, and action. *Am. J. Med.*, **85**, Suppl. 5A, 44–58.

FÖRSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849–1857.

GERICH, J.E., CHARLES, M.A. & GRODSKY, G.M. (1974). Characterization of the effects of arginine and glucose on glucagon and insulin release from the perfused rat pancreas. *J. Clin. Invest.*, **54**, 833–841.

GOTOH, M., MAKI, T., KIYOIZUMI, T., SATOMI, S. & MONACO, A.P. (1985). An improved method for isolation of mouse pancreatic islets. *Transplantation*, **40**, 437–438.

HARPER, J.F. & BROOKER, G. (1975). Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O-acetylation by acetic anhydride in aqueous solution. *J. Cycl. Nucl. Res.*, **1**, 207–218.

HELLMAN, B., IDAHL, L.-Å., LERNMARK, Å., SEHLIN, J. & TÄLJEDAL, I.-B. (1974). Membrane sulfhydryl groups and pancreatic beta cell recognition of insulin secretagogues. *Excerpt. Med. Int. Congr. Ser.*, **312**, 65–78.

HOGG, N., DARLEY-USMAR, V.M., WILSON, M.T. & MONCADA, S. (1992). Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem. J.*, **281**, 419–424.

JANSSON, L. & SANDLER, S. (1991). The nitric oxide synthase inhibitor II N^G-nitro L-arginine stimulates pancratic islet insulin release in vitro, but not in the perfused pancreas. *Endocrinology*, **128**, 3081–3085.

JONES, P.M., PERSAUD, S.J., BJAALAND, T., PEARSON, J.D. & HOWELL, S.L. (1992). Nitric oxide is not involved in the initiation of insulin secretion from rat islets of Langerhans. *Diabetologia*, **35**, 1020–1027.

LAYCHOCK, R.G., MODICA, M.E. & CAVANAUGH, C.T. (1991). L-Arginine stimulates cyclic guanosine 3',5'-monophosphate formation in rat islets of Langerhans and RIN m5F insulinoma cells: evidence for L-arginine: nitric oxide synthase. *Endocrinology*, **129**, 3043–3052.

LUNDQUIST, I., PANAGIOTIDIS, G. & STENSTRÖM, A. (1991). Effect of L-DOPA administration on islet monoamine oxidase activity and glucose-induced insulin release in the mouse. *Pancreas*, **6**, 522–527.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

ORRENIUS, S. & MOLDÉUS, P. (1984). The multiple roles of glutathione in drug metabolism. *Trends. Pharmacol. Sci.*, **5**, 432–435.

PANAGIOTIDIS, G., ALM, P. & LUNDQUIST, I. (1992). Inhibition of islet nitric oxide synthase increases arginine-induced insulin release. *Eur. J. Pharmacol.*, **229**, 277–278.

PANAGIOTIDIS, G., LINDSTRÖM, P., STENSTRÖM, A. & LUNDQUIST, I. (1993a). Glucose modulation of islet monoamine oxidase activity in lean and obese hyperglycemic mice. *Metabolism*, **42**, 1398–1404.

PANAGIOTIDIS, G., STENSTRÖM, A. & LUNDQUIST, I. (1993b). Influence of β_2-Adrenoceptor stimulation and glucose on islet monoamine oxidase activity and insulin secretory response in the mouse. *Pancreas*, **8**, 368–374.

PANAGIOTIDIS, G., STENSTRÖM, A. & LUNDQUIST, I. (1993c). Effects of adrenergic and cholinergic stimulation on islet monoamine oxidase activity and insulin secretion in the mouse. *Eur. J. Pharmacol.*, **233**, 285–290.

PANAGIOTIDIS, G., STENSTRÖM, A. & LUNDQUIST, I. (1994). *In vivo* action of cyclic AMP modulating secretagogues on islet monoamine oxidase activity and insulin release. *Endocrine*, **2**, 571–576.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br. J. Pharmacol.*, **101**, 325–328.

REES, D.D., PALMER, R.M.J., SHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

SALEHI, A. & LUNDQUIST, I. (1993). CA^{2+} deficiency, selective α-glucosidehydrolase inhibition, and insulin secretion. *Am. J. Physiol.*, **265**, E1–E9.

SCHMIDT, H.H.H.W., WARNER, T.D., ISHII, K., SHENG, H. & MURAD, F. (1992). Insulin secretion from pancreatic B cells caused by L-arginine-derived nitrogen oxides. *Science*, **255**, 721–723.

STAMLER, J.S., SIMON, D.I., OSBORNE, J.A., MULLINS, M.E., JARAKI, O., MICHEL, T., SINGEL, D.J. & LOSCALZO, J. (1992). S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 444–448.

STEINER, A.L., PARKER, C.W. & KIPNIS, D.M. (1972). Radioimmunoassay for cyclic nucleotides. *J. Biol. Chem.*, **247**, 1106–1120.

VARA, E. & TAMARIT-RODRIGUEZ, J. (1991). Does cyclic guanosine monophosphate mediate noradrenaline-induced inhibition of islet insulin secretion stimulated by glucose and palmitate? *Biochem. J.*, **278**, 243–248.

VINCENT, S.R. (1992). Nitric oxide and arginine-evoked insulin secretion. *Science*, **258**, 1376–1378.

WELSH, N., EIZIRIK, D.L., BENDTZEN, K. & SANDLER, S. (1991). Interleukin-1b-induced nitric oxide production in isolated rat pancreatic requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology*, **129**, 3167–3173.

(Received October 15, 1993
Revised June 22, 1994
Accepted September 15, 1994)