Arginine-induced insulin release is decreased and glucagon increased in parallel with islet NO production

Ragnar Henningsson and Ingmar Lundquist. Arginine-induced insulin release is decreased and glucagon increased in parallel with islet NO production. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E500–E506, 1998.—Nitric oxide (NO) produced by islet constitutive NO synthase (cNOS) is a putative modulator of islet hormone secretion. We show here for the first time that the release of insulin induced by L-arginine or L-homoarginine is inhibited and that of glucagon stimulated in parallel with the rate of islet NO production. It was found that L-homoarginine was ≈25–30% less potent than L-arginine as an insulin secretagogue but equally potent as a glucagon secretagogue. Biochemical determination of islet cNOS activity revealed that the NO production with L-homoarginine as substrate was only ≈40% of that of L-arginine. Selective inhibition of islet cNOS potentiated insulin release during amino acid stimulation. Moreover, inhibition of cNOS suppressed glucagon release, more so with L-arginine than with L-homoarginine as secretagogue, reflecting the relative rates of their NO production. In K<sup>+</sup>-depolarized islets, inhibition of cNOS enhanced the insulin response to L-arginine by 50% and that to L-homoarginine by 23%, largely corresponding to their relative NO production. The intracellular NO donor hydroxylamine dose dependently inhibited insulin but increased glucagon secretion in K<sup>+</sup>-depolarized and amino acid-stimulated islets. We conclude that both amino acids have a dual action on insulin release, since their stimulatory effects are reduced in parallel with the rates of their NO production. Furthermore, the greater NO production induced by L-arginine relative to L-homoarginine corresponds to NO-mediated increases in glucagon release. These NO effects are mainly exerted independently of membrane depolarization events.

Islets of Langerhans; L-arginine; L-homoarginine; nitric oxide synthase activity; insulin secretion; glucagon secretion; nitric oxide synthase inhibitors

**L-ARGININE STIMULATES insulin release.** Three different mechanisms have been suggested to account for this stimulation (6, 38): 1) β-cell uptake of the positively charged L-arginine molecule followed by depolarization of the plasma membrane; 2) L-arginine metabolism through the action of arginase (L-arginine is thereby hydrolyzed to urea and ornithine, and ornithine is then further metabolized, ending up in the citric acid cycle); and 3) stimulation by nitric oxide (NO) derived from the metabolism of L-arginine through the action of a constitutive NO synthase (cNOS). The metabolism of L-arginine through this latter pathway yields equimolar concentrations of NO and L-citrulline (23, 36, 38). Exactly how NO influences islet hormone secretion is still rather controversial, however, and both stimulatory (26, 38) and inhibitory (2, 3, 17, 21, 31–33, 36) effects on insulin release have been reported. Already in 1992, we observed that the NO synthase (NOS) inhibitor NG<sup>+</sup>-nitro-L-arginine potentiated L-arginine-induced insulin release from isolated mouse islets (33). These data prompted us to suggest that NO, evolved from L-arginine-induced islet cNOS activity, restrained insulin secretion stimulated by L-arginine and thus that L-arginine had a dual action on insulin release (33). Later on we found that NO, in contrast, exerted a stimulatory effect on glucagon secretion (31).

It has been our aim in the present study to further investigate the complex influence of NO within the islets of Langerhans with special regard to its action on L-arginine-induced hormone release. Because L-homoarginine, a close analog to L-arginine, is known to be poorly metabolized by NOS activities in other tissues (22), we have compared the effects of L-arginine with those of L-homoarginine concerning islet NO production in relation to the relative potency of the amino acids to induce insulin and glucagon secretion. A new microtechnique for NOS assay in small tissue samples (11, 36) was used to perform a direct biochemical determination of cNOS activity in islet homogenates. Furthermore, two different NOS inhibitors (7, 35), N<sup>6</sup>-monomethyl-L-arginine (L-NMMA) and N<sup>2</sup>-nitro-L-arginine methyl ester (L-NAME), were employed when we studied the consequences of inhibition of cNOS activity on islet hormone release. Aside from its NOS inhibitory properties, L-NAME, at least in high concentrations, has recently been reported to induce closure of ATP-sensitive K<sup>+</sup> channels in the β-cells (25). It was therefore important to compare the effects of L-NAME with those of L-NMMA, since L-NMMA has not been reported to exert this effect. To avoid interference by membrane depolarization events, some experiments were performed with islets exposed to diazoxide, a known K<sup>+</sup>-channel opener (27), in combination with a depolarizing concentration of K<sup>+</sup> (14) in the absence and presence of L-NAME or L-NMMA. In addition, we tested the effects of the intracellular NO donor hydroxylamine on insulin and glucagon secretion from K<sup>+</sup>-depolarized islets.

**MATERIALS AND METHODS**

Animals. Female mice of the NMRI strain (B&K, Sollentuna, Sweden) weighing 25–30 g were used in all studies. They were fed a standard pellet diet (B&K) and tap water ad libitum. The experiments were approved by the Ethical Committee for Animal Research at Lund University.

Drugs and chemicals. Collagenase (CLS4) was obtained from Worthington Biochemicals (Freehold, NJ). L-NMMA, L-NAME, and hydroxylamine as well as L-arginine and L-homoarginine were from Sigma (St. Louis, MO). BSA was from ICN Biochemicals (High Wycombe, UK). All other chemicals were from Merck (Darmstadt, Germany). The RIA kits for insulin and glucagon determination were obtained from Diagnostika (Falkenberg, Sweden) and Euro-Diagnostics (Malmö, Sweden), respectively.
Assay of islet NOS. Preparation of isolated pancreatic islets from the mouse was performed by retrograde injection of a collagenase solution via the bile-pancreatic duct (16). Islets were then isolated and handpicked under a stereomicroscope at room temperature. The freshly isolated islets were then thoroughly washed and collected in ice-cold buffer (200 islets in 840 µl buffer) containing 20 mM HEPES, 0.5 mM EDTA, and 1 mM N2-dithiothreitol, pH 7.2, and immediately frozen at −20°C. On the day of assay, the islets were sonicated on ice, and the buffer solution containing the islet homogenate was supplemented to also contain 0.45 mM CaCl2, 2 mM NADPH, 25 mM L-calmodulin, and 0.2 mM L-arginine or 0.2 mM L-homoarginine in a total volume of 1 ml. The buffer composition is essentially the same as previously described for assay of NOS in brain tissue using radiolabeled L-arginine (10). The homogenate was then incubated at 37°C under constant air bubbling, 1.0 ml/min, for 3 h. It was ascertained that under these conditions, the reaction velocity was linear for at least 6 h. Aliquots of the incubated homogenate (200 µl) were then passed through a 1-ml Amprep CBA cation-exchange column for HPLC analysis. The amount of L-citrulline (or L-homocitrulline) formed was then measured in a Hitachi F 1000 fluorescence spectrophotometer (Merck) as previously described (36). NO and citrulline are produced in equimolar concentrations. The methodology has been described in detail earlier (11, 36), the only difference being that the incubation was now performed at 37°C instead of at room temperature (36). Protein was determined according to Bradford (9) on samples from the original homogenate.

Hormone secretion. Freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer (24), pH 7.4, supplemented with 10 mM HEPES, 0.1% BSA, and 1 mM glucose. Each incubation vial contained 10 islets in 1.0 ml of buffer solution and was gassed with 95% O2-5% CO2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium containing 7 mM glucose plus or minus the different test agents, and then the islets were incubated for 60 min. All incubations were performed at 37°C in a shaking water bath (30 cycles/min). Immediately after incubation, aliquots of the medium were removed and frozen for subsequent assay of insulin and glucagon (1, 18, 34).

Statistics. Statistical significance between sets of data was assessed using unpaired Student’s t-test or, where applicable, ANOVA followed by Tukey-Kramer’s multiple comparisons test. Results are expressed as means ± SE.

RESULTS

cNOS activity in islet homogenates with L-arginine or L-homoarginine as substrate. In the first series of experiments, we analyzed the production of NO (measured as L-citrulline or L-homocitrulline) in mouse islet homogenates after incubation with the different NOS substrates at enzyme-saturated conditions. At 37°C, the islet cNOS enzyme with L-arginine as substrate displayed an activity of 29.6 ± 0.90 pmol L-citrulline·mg protein·h−1 (n = 6). When L-arginine was replaced by L-homoarginine as substrate, the NO production was only 11.3 ± 0.83 pmol L-homocitrulline·mg protein·h−1 (n = 5), i.e., only 40% of that of L-arginine. In the absence of Ca2+/calmodulin or substrate, the islet homogenates were almost devoid of cNOS activity.

Dose-response relationship for L-arginine and L-homoarginine on islet hormone release. Figure 1A illustrates a dose-response curve showing the stimulatory effect of L-arginine and L-homoarginine on insulin release. The maximal stimulatory concentration for insulin release was reached at ~15 mM for L-arginine (20 mM giving the same response; data not shown in the graph) and at ~10 mM for L-homoarginine. At concentration levels of 5 and 10 mM, L-homoarginine was ~25% less potent than L-arginine. At a 15 mM concentration, the L-homoarginine-induced release was 40% less than that induced by L-arginine. Glucagon secretion (Fig. 1B) was also dose dependently stimulated by L-arginine and L-homoarginine. In this case, the two amino acids showed exactly the same potency.
Effects of \( L\)-NAME and \( L\)-NMMA on islet hormone release stimulated by \( L\)-arginine or \( L\)-homoarginine. The first set of experiments was designed to study the effects of the NOS inhibitors \( L\)-NAME and \( L\)-NMMA on the secretion of insulin and glucagon from isolated islets at a basal physiological glucose concentration (7 mM). The two NOS inhibitors showed no effects at all either on basal insulin or on basal glucagon secretion at a 5 mM concentration in the presence of 7 mM glucose [i.e., insulin controls 0.33 ± 0.020 ng insulin·islet\(^{-1}·h^{-1}\) \((n = 12)\) vs. 0.28 ± 0.044 \((n = 8)\) in the presence of \( L\)-NAME and 0.33 ± 0.045 \((n = 8)\) in the presence of \( L\)-NMMA]. The results for glucagon were as follows: glucagon controls 26.0 ± 1.81 pg glucagon·islet\(^{-1}·h^{-1}\) vs. 21.2 ± 2.12 (\( L\)-NAME) and 25.0 ± 2.50 (\( L\)-NMMA). A concentration of 5 mM of the inhibitors was chosen, since previous dose-response studies (32) showed that this was the highest concentration of \( L\)-NAME that did not per se affect insulin release.

Figure 2 illustrates that addition of either of the two NOS inhibitors to an incubation medium containing \( L\)-arginine (10 mM) or \( L\)-homoarginine (10 mM) did increase insulin secretion (Fig. 2, A and C) but decreased glucagon secretion (Fig. 2, B and D). The effect of \( L\)-NAME was greater than that of \( L\)-NMMA concerning both \( L\)-arginine- and \( L\)-homoarginine-stimulated insulin secretion. It should be noted that insulin secretion in response to \( L\)-homoarginine + \( L\)-NAME exceeded that for \( L\)-arginine + \( L\)-NAME, although \( L\)-arginine is a more potent insulin secretagogue than \( L\)-homoarginine. No such differences could be seen with regard to glucagon secretion (Fig. 2, B and D). Furthermore, the effect of \( L\)-homoarginine as insulin secretagogue in this series of experiments was again less than that of \( L\)-arginine (=30\%) (Fig. 2, A and C).

Effects of \( L\)-NAME and \( L\)-NMMA on islet hormone release stimulated by \( L\)-arginine or \( L\)-homoarginine in the presence of diazoxide and a depolarizing concentration of \( K^+\). Figure 3 shows a strong stimulatory effect by \( KCl\) (30 mM) in the presence of diazoxide (250 \( \mu\)M) on basal insulin and glucagon secretion at 7 mM glucose. No significant effects on insulin release were found when either \( L\)-NAME or \( L\)-NMMA was added (Fig. 3A). In contrast, we observed a modest suppressive action by both NOS inhibitors on glucagon release (Fig. 3B).

Figure 4, A and C, shows insulin secretion stimulated by \( L\)-arginine and \( L\)-homoarginine in the presence of \( K^+\) and diazoxide. Addition of \( L\)-NAME or \( L\)-NMMA further enhanced \( L\)-arginine-stimulated insulin release by ~50\% and \( L\)-homoarginine-stimulated insulin release by ~25\%, i.e., the effects being largely in accordance with the relative rate of NO production. Furthermore, in the presence of \( K^+\) and diazoxide, we found no apparent difference between the effects of the two NOS inhibitors \( L\)-NAME and \( L\)-NMMA on insulin release stimulated by either \( L\)-arginine or \( L\)-homoarginine. This was in contrast to what we observed in the absence of \( K^+\) and diazoxide (cf. Fig. 2, A and C).

Figure 4, B and D, shows that addition of \( K^+\) and diazoxide also strongly increased glucagon secretion stimulated by \( L\)-arginine or \( L\)-homoarginine. Moreover, addition of \( L\)-NAME and \( L\)-NMMA suppressed glucagon
 secretion induced by both amino acids, the effect of L-NAME being slightly more pronounced than that of L-NMMA. It should be noted (Fig. 4, A and C) that the insulin response to L-arginine was still very prominent, whereas that to L-homoarginine was almost abolished compared with the effect of K+ + diazoxide alone (=4 ng insulin/islet h−1), being recorded in the same series of experiments (cf. Fig. 3A).

Influence of the intracellular NO donor hydroxylamine on islet hormone release induced by L-arginine or L-homoarginine in the presence of diazoxide, L-NAME, and a depolarizing concentration of K+. Figure 5 illustrates the dose-response pattern induced by the intracellular NO donor hydroxylamine on insulin and glucagon release stimulated by L-arginine (Fig. 5, A and B) or L-homoarginine (Fig. 5, C and D) in the presence of diazoxide, L-NAME, and a depolarizing K+ concentration. The NO donor, at 0.003–3.0 mM, dose dependently inhibited insulin release induced by both amino acids (Fig. 5, A and C). In contrast, glucagon secretion was potentiated. Only concentrations of 0.003 and 0.03 mM of the drug increased glucagon secretion (Fig. 5, B and D).

DISCUSSION

In the present study, we found that the NO evolution when L-homoarginine was used as a substrate was only ~40% of that produced by L-arginine. This is in good agreement with measurements in other tissues (19, 22, 30) and thus suggests, together with our previous data (36), that islet cNOS shares the properties described for the Ca2+-calmodulin-regulated isoforms in the brain and in the vascular endothelium (23). It should be noted that the recorded cNOS activity with L-arginine as a substrate in our present investigation was approximately four times higher compared with our previous experiments, in which the homogenate incubations were performed at room temperature (36).

Insulin secretion. Equimolar concentrations of L-arginine and L-homoarginine (10 mM; being maximal with regard to islet hormone release for the latter amino acid) induced exactly the same response with regard to insulin release, whereas the insulin response to L-arginine was ~25–30% greater than that to L-homoarginine. The insulin response to L-arginine has been attributed to intracellular accumulation of this positively charged amino acid, whereby the plasma membrane is depolarized and Ca2+ influx is initiated (21). This property is shared by L-homoarginine, which is taken up by the same transport system, (y)⁺, and also carries a net positive charge equal to that of L-arginine (6, 20, 37). Thus quantitative differences in the insulin-releasing action between these amino acids cannot be accounted for by the cationic charge. L-Homoarginine is reportedly not metabolized by arginase within islet tissue (28), and because L-arginine metabolism is another proposed mechanism for the stimulatory effect of this amino acid on insulin release (28), the more efficient “fuel properties” of L-arginine, as well as its ability to increase cAMP (32), may explain why L-arginine is a more potent insulin secretagogue than its close analog L-homoarginine. This hypothesis is further strengthened by our present finding that the insulin-releasing effect of L-homoarginine was almost abolished in depolarized islets, thus indicating that the main mechanism of action of L-homoarginine to stimulate insulin release is intimately coupled to membrane depolarization events. Our observation that L-arginine is a more efficient insulin secretagogue than L-homoarginine at an amino acid concentration of 10 mM (being maximal for L-homoarginine) is at variance with previous data from rat islets, where no such difference was found (6). The explanation for this discrepancy is presently unclear.

The potentiating effects of the two NOS inhibitors (L-NAME and L-NMMA) on the amino acid-stimulated insulin release were not identical. L-NMMA potenti ated L-arginine- and L-homoarginine-stimulated insulin secretion much less than L-NAME. Because L-NAME per se in very high concentrations (>5 mM) reportedly has significant effects on β-cell electrical activity and ion-channel currents (13, 25), a modest effect of L-NAME on the ATP-sensitive K+ channels (KATP channels) is in accordance with our present data. Moreover, we could...
show that the effects of L-NAME on β-cells already fully depolarized by K⁺ (30 mM) and treated with diazoxide to keep the K⁺ channels open were significantly reduced and almost identical to those of L-NMMA with regard to insulin release induced by both amino acids. In fact, by taking the potentiating effects of the two NOS inhibitors on L-arginine-induced insulin release in K⁺-depolarized islets as 100%, the same effect on L-homoarginine-induced insulin release was only ≈40% of that of L-arginine, i.e., in almost perfect agreement with the relative rate of NO production from the two cNOS substrates. We therefore interpret the effects of 5 mM L-NAME on L-arginine- and L-homoarginine-stimulated insulin secretion as being due to a combination of K<sub>ATP</sub>-channel closure and inhibition of cNOS activity.

Fig. 4. Effect of NOS inhibitors L-NAME (5 mM) and L-NMMA (5 mM) after membrane depolarization (KCl 30 mM) and K⁺-channel opening (diazoxide 250 µM) on L-arginine (10 mM)-stimulated insulin (A) and glucagon (B) secretion and on L-homoarginine (10 mM)-stimulated insulin (C) and glucagon (D) secretion from isolated islets. Glucose concentration was 7 mM. Values are means ± SE. There were 8–12 batches of islets in each group. Each batch (incubation vial) consisted of 10 islets in 1 ml of incubation medium. Incubation time was 60 min. Results are from 3 separate experiments run on different days. Significant differences vs. L-arginine or L-homoarginine controls (open bars) in presence of KCl and diazoxide: *P < 0.05, **P < 0.01, and ***P < 0.001.

Fig. 5. Effect of different concentrations of intracellular nitric oxide donor hydroxylamine on insulin (A and C) and glucagon (B and D) release from isolated islets stimulated by 10 mM L-arginine (A and B) or 10 mM L-homoarginine (C and D) in presence of 7 mM glucose + 30 mM KCl + 250 µM diazoxide + 5 mM L-NAME. Values are means ± SE. There were 10–12 batches of islets in each group. Each batch (incubation vial) consisted of 10 islets in 1 ml of incubation medium. Incubation time was 60 min. Results are from 4 separate experiments run on different days. Significant differences vs. L-arginine or L-homoarginine controls: **P < 0.01 and ***P < 0.001.
Finally, we observed in K⁺-depolarized islets that the intracellular NO donor hydroxylamine could mimic the inhibitory effect on insulin release elicited by endogenous NO in the presence of either of the two amino acids. Because the experiments were conducted in the presence of L-NAME, they also indicated that the artificially produced NO, delivered by the NO donor, could overcome the cNOS inhibitory action of L-NAME and that L-NAME had no direct effects on NO itself. It should be noted that recent data (4) have shown that hydroxylamine can inhibit glucose-induced insulin release by activating K_ATP channels. This observation can now be further extended, since our present results indicate that hydroxylamine-derived NO has additional important inhibitory effects on insulin secretory processes, being exerted independently of membrane depolarization events.

Glucagon secretion. L-Arginine and L-homoarginine were equally potent in stimulating glucagon secretion. Hence, the effect of L-arginine to stimulate glucagon release is probably independent of any “fuel effect” exerted by this amino acid in the glucagon cell. Therefore, depolarization with subsequent Ca²⁺ influx is conceivably responsible for the main glucagon-releasing action of both amino acids. Furthermore, in contrast to their effects on insulin release, both NOS inhibitors, i.e., L-NMMA and L-NAME, did not stimulate but inhibited glucagon release induced by both L-arginine and L-homoarginine. Moreover, this inhibition was quantitatively the same irrespective of whether L-NMMA or L-NAME was used. This is in accordance with the notion that the glucagon-producing cells are lacking K_ATP channels (5). Thus L-NAME was found to inhibit L-arginine-stimulated glucagon secretion by 27% compared with the effect of L-NMMA (being 31%). Regarding glucagon secretion stimulated by L-homoarginine, the corresponding values were 18% reduction (L-NAME) and 21% reduction (L-NMMA). The greater inhibition of cNOS activity by the two NOS inhibitors in the presence of L-arginine than in the presence of L-homoarginine corresponded to the comparatively greater NO evolution derived from L-arginine than from L-homoarginine.

The present data showing that the NOS inhibitors greatly reduced glucagon release stimulated by L-arginine and L-homoarginine in depolarized islets suggest that this effect is largely independent of membrane depolarization events and thus probably exerted at a later step in the stimulus-secretion coupling. Such an assumption was moreover evidenced by our observation that the intracellular NO donor hydroxylamine could further increase glucagon release already stimulated by maximal doses of both amino acids in depolarized islets in the presence of L-NAME. These data thus suggest that NO may serve as an important messenger of glucagon release. Incidentally, as mentioned above, a maximal glucagon-releasing dose of L-homoarginine induced the same response as a maximal dose of L-arginine, yet the rate of NO production from L-homoarginine was only 40% of that of L-arginine. Thus L-arginine would be expected to bring about a greater glucagon release than L-homoarginine. It is not inconceivable that this greater NO-mediated glucagon release induced by L-arginine could have been counteracted, and thus masked, by an inhibitory fuel effect of this arginase-metabolized amino acid. That would be in line with the well-known “fuel”-induced inhibition of glucagon release exerted by glucose.

Concerning the NO effects vs. the electrogenic effects induced by the two amino acids on the secretory processes of both insulin and glucagon, available evidence indicates that cNOS activity is fully saturated at a concentration of 0.2 mM for both amino acids (Refs. 11 and 36 and unpublished data), whereas concentrations of 3–5 mM are required to stimulate islet hormone secretion (cf. Fig. 1). Hence, the NO-mediated effects are apparently exerted at an approximately ten times lower concentration of the cationic amino acids than their major hormone-releasing action, which evidently is exerted through membrane depolarization and Ca²⁺ influx.

In addition, the question arises as to whether the effects of NO are elicited solely within the cell of origin or are also exerted through the paracrine route. There is a general agreement that cNOS activity is located at the insulin-producing β-cells (8, 12, 31, 33, 38), whereas there are conflicting reports on its occurrence in the glucagon cells (8, 12). Because insulin is reportedly an inhibitor of glucagon release (29), it cannot be excluded that a decreased/increased insulin release might contribute to an increased/decreased glucagon release. However, in several of our present experiments, there is either a lack of or a very poor correlation between these parameters, and thus there is reason to believe that the main effects are in fact mediated by NO itself either within the cell of origin and/or through the paracrine route.

Conclusion. We conclude that NO is produced within the islets of Langerhans and that L-arginine is a much better substrate for islet cNOS than its analog L-homoarginine. Both amino acids have a dual action on insulin release, since their stimulatory actions are reduced in relation to the rate of their NO production. Moreover, the stimulatory electrogenic action of the two amino acids on glucagon release is further increased by the relative rates by which they produce NO. The intracellular NO donor hydroxylamine inhibited insulin and stimulated glucagon release in K⁺-depolarized islets, thus mimicking the effects of islet endogenous NO production. Hence NO is a negative modulator of insulin release and a positive modulator of glucagon release induced by L-arginine or L-homoarginine. These NO effects are mainly exerted independently of membrane depolarization events.

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