Endocrine, *vol. 16, no. 2, 97–104, November 2001* 0969–711X/01/16:97–104/$12.00

Total Parenteral Nutrition Modulates Hormone Release by Stimulating Expression and Activity of Inducible Nitric Oxide Synthase in Rat Pancreatic Islets

Albert Salehi,[1] Mats Ekelund,[2] Ragnar Henningsson,[1] and Ingmar Lundquist[1]

Institute of Physiological Sciences, Departments of [1]Pharmacology and [2]Surgery, University of Lund, Sweden

The expression and activities of constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS) in relation to insulin and glucagon secretory mechanisms were investigated in islets isolated from rats subjected to total parenteral nutrition (TPN) for 10 d. TPN is known to result in significantly increased levels of plasma lipids during the infusion time. In comparison with islets from freely fed control rats, islets taken from TPN rats at d 10 displayed a marked decrease in glucose-stimulated insulin release (4.65 ± 0.45 ng/[islet·h] vs 10.25 ± 0.65 for controls) ($p < 0.001$) accompanied by a strong iNOS activity (18.3 ± 1.1 pmol of NO/[min·mg of protein]) and a modestly reduced cNOS activity (11.3 ± 3.2 pmol of NO/[min.mg of protein] vs 17.7 ± 1.7 for controls) ($p < 0.01$). Similarly, Western blots showed the expression of iNOS protein as well as a significant reduction in cNOS protein in islets from TPN-treated rats. The enhanced NO production, which is known to inhibit glucose-stimulated insulin release, was manifested as a strong increase in the cyclic guanosine 5'-monophosphate content in the islets of TPN-treated rats (1586 ± 40 amol/islet vs 695 ± 64 [$p < 0.001$] for controls). Moreover, the content of cyclic adenosine monophosphate (cAMP) was greatly increased in the TPN islets (80.4 ± 2.1 fmol/islet vs 42.6 ± 2.6 [$p < 0.001$] for controls). The decrease in glucose-stimulated insulin release was associated with an increase in the activity of the secretory pathway regulated by the cAMP system in the islets of TPN-treated rats, since the release of insulin stimulated by the phosphodiesterase inhibitor isobutylmethylxanthine was greatly increased both in vivo after iv injection and after in vitro incubation of isolated islets. By contrast, the release of glucagon was clearly reduced in islets taken from TPN-treated rats (33.5 ± 1.5 pg/[islet·h] vs 45.5 ± 2.2 for controls) ($p < 0.01$) when islets were incubated at low glucose (1.0 mmol/L). The data show that long-term TPN treatment in rats brings about impairment of glucose-stimulated insulin release, that might be explained by iNOS expression and a marked iNOS-derived NO production in the β-cells. The release of glucagon, on the other hand, is probably decreased by a direct "nutrient effect" of the enhanced plasma lipids. The results also suggest that the islets of TPN-treated rats have developed compensatory insulin secretory mechanisms by increasing the activity of their β-cell cAMP system.

Key Words: Pancreatic islets; insulin secretion; glucagon secretion; inducible nitric oxide synthase.

Introduction

Nitric oxide (NO) is now accepted as an ubiquitous messenger molecule in different organ systems, and also as an important defense mechanism against invading microorganisms *(1,2)*. The messenger function is accomplished mainly by NO derived from two isoforms of the constitutive NO synthase (cNOS): neuronal cNOS (ncNOS) and endothelial cNOS. These enzymes are Ca^{2+}/calmodulin dependent. The third isoform has been mainly implicated in different defense mechanisms of the body and is labeled inducible NOS (iNOS). This isoform is Ca^{2+}/calmodulin independent and known to be induced by cytokines and various inflammatory agents *(1,2)*. We and others have shown the morphologic substrate for the occurrence of both ncNOS and iNOS in the islets of Langerhans *(3–7)*. NO derived from islet ncNOS activity is implicated as a regulator of insulin and glucagon release, and most data to date suggest that NO inhibits glucose-stimulated insulin release and serves as a positive modulator of glucagon release *(6,8–18)*. NO derived from iNOS, on the other hand, has been assigned an important role in the autoimmune destruction and dysfunction of β-cells in insulin-dependent diabetes mellitus (cf. refs. *19–21*). Thus, after cytokine stimulation, iNOS-derived NO can be produced in islet tissue both from invading macrophages and from the β-cells themselves *(19–21)*.

Received June 21, 2001; Revised October 5, 2001; Accepted October 8, 2001.

Author to whom all correspondence and reprint requests should be addressed: Dr. Albert Salehi, Institute of Physiological Sciences, Department of Pharmacology, BMC F13, S-221 84 Lund, Sweden. E-mail: Salehi@farm.lu.se

Recently, we have obtained evidence for a "noncytokine"-activated iNOS activity in islet tissue during in vitro "hyperglycemic" conditions *(18)*. The data suggested to us that iNOS-derived NO could be involved in β-cell failure also during the development of noninsulin-dependent diabetes (NIDDM), since high glucose was found to activate and unmask a latent iNOS activity in healthy mouse islets *(18)*. In addition to "glucotoxicity,"—hyperglycemia as a putative contributing factor in the pathogenesis of β-cell failure in NIDDM—"lipotoxicity," owing to elevated plasma lipids, has been suggested as an important factor in this context *(22)*. A possible involvement of elevated plasma lipids in β-cell failure was previously reported *(22,23)*, and recently we observed a markedly impaired insulin response to iv glucose in rats subjected to total parenteral nutrition (TPN) *(24)*. This was especially interesting because patients undergoing TPN have elevated plasma lipids and often display a disturbed carbohydrate tolerance *(25)*. We have previously elaborated a TPN solution for rat purposes with components similar to those used in clinical practice in order to have an animal model in which the metabolic effects of TPN can be studied in more detail *(24,26)*. The aim of the present investigation was to study the effects of long-term TPN infusion in rats on glucose-stimulated insulin release from isolated islets in relation to the activity and protein expression of cNOS as well as iNOS in islets taken from such animals.

Results

There was no difference in body weights of controls and TPN rats at the end of the experiments. Further, there was no difference in plasma glucose levels between controls and TPN rats either before, during, or at the end of the TPN infusion (data not shown).

Effects of TPN Infusion on Hormone Secretion from Freshly Isolated Islets Incubated in Presence of Low and High Glucose

Figure 1 shows the effect of low (1 mmol/L) and high (16.7 mmol/L) glucose on insulin and glucagon release from islets isolated from either freely fed (FF) controls or TPN-treated rats. Insulin release from TPN islets was slightly increased at low glucose but markedly suppressed at high glucose. By contrast, glucagon release at low glucose (1.0 mmol/L), when the rate of glucagon secretion is high, was markedly reduced in TPN islets but similar to FF controls at high glucose, which by itself is known to suppress glucagon release.

Influence of TPN on Islet NOS Activities

As seen in Fig. 2A, TPN treatment brought about a strong upregulation of islet iNOS activity. As expected, no iNOS activity could be detected in FF control animals. Moreover, islet cNOS activity was decreased by TPN treatment. However, because of the marked increase in iNOS activity, total

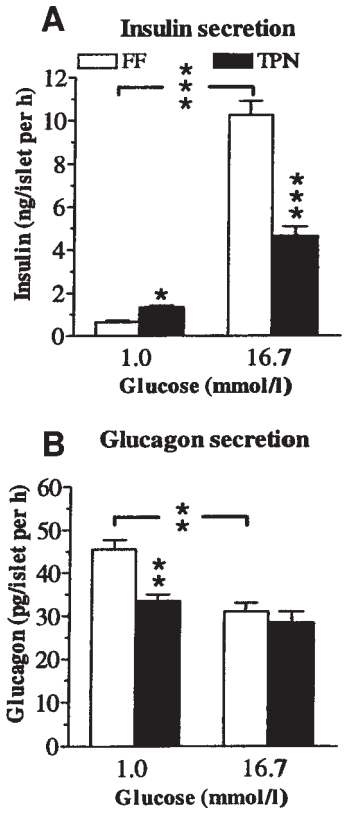

Fig. 1. Basal (1.0 mmol/L of glucose) and glucose-stimulated (16.7 mmol/L) release of (**A**) insulin and (**B**) glucagon in islets isolated from FF controls and TPN-treated rats and incubated for 60 min. The means ± SEM for five to nine batches of islets each isolated from a single rat are shown. Asterisks denote the probability level of random difference for islets from FF vs TPN-treated rats and for differences between 1.0 and 16.7 mmol/L of glucose. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

NOS activity was clearly enhanced in these islets (Fig. 2A). The induction of iNOS after TPN infusion was confirmed by Western blot analysis showing that visually there was an exclusive expression of iNOS in the pancreatic islets isolated from TPN-treated rats (Fig. 2B). This was confirmed by densitometric analysis, which showed that iNOS expression increased from 0.30 ± 0.045 (FF controls) to 9.27 ± 0.83 units (TPN) ($p < 0.001$) (FF controls: $n = 3$; TPN: $n = 3$). Moreover, the biochemical observation (Fig. 2A) of a decreased cNOS activity in TPN islets was reflected in a significant reduction in the expression of cNOS protein from 17.76 ± 2.11 (FF controls) to 10.21 ± 1.51 units (TPN) ($p < 0.01$) (FF controls: $n = 4$; TPN: $n = 4$) (Fig. 2B).

Influence of TPN Treatment on Islet Content of Cyclic Guanosine 5'-Monophosphate and Cyclic Adenosine Monophosphate

Since we recently found that NO-induced impairment of glucose-stimulated insulin release in mice was associated with upregulation of the islet cyclic adenosine monophosphate (cAMP) system *(18)*, we measured the content

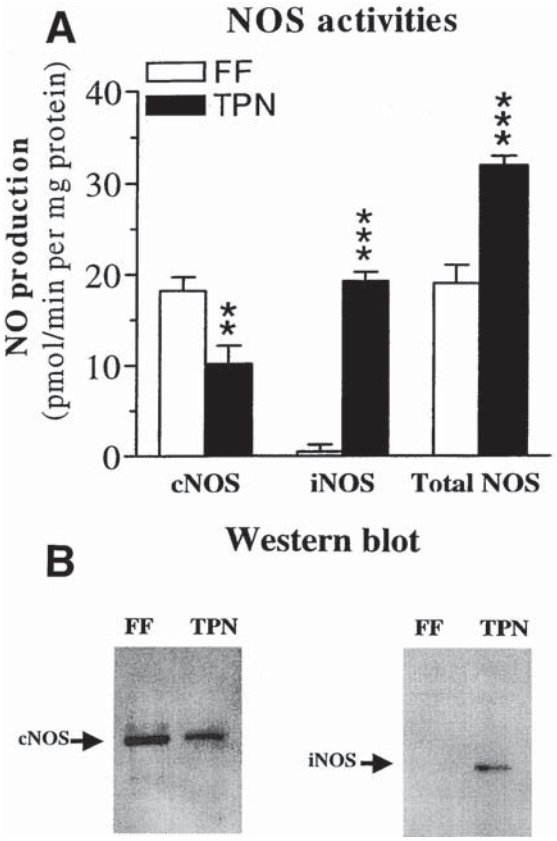

Fig. 2. (**A**) cNOS, iNOS, and total NOS activities measured as L-citrulline formation (pmol/[min·mg protein]) in islets isolated from FF controls and TPN-treated rats.The means ± SEM for islets from six rats in each group are shown. Each batch was isolated from a single rat. $^{**}p < 0.01$; $^{***}p < 0.001$. (**B**) Representative example of Western blots of islets taken from FF or TPN-treated rats and incubated with cNOS or iNOS antibody. The blots were performed with 20 µg of islet protein on each lane. Arrows indicate the molecular weight of 150 (cNOS) and 130 kDa (iNOS).

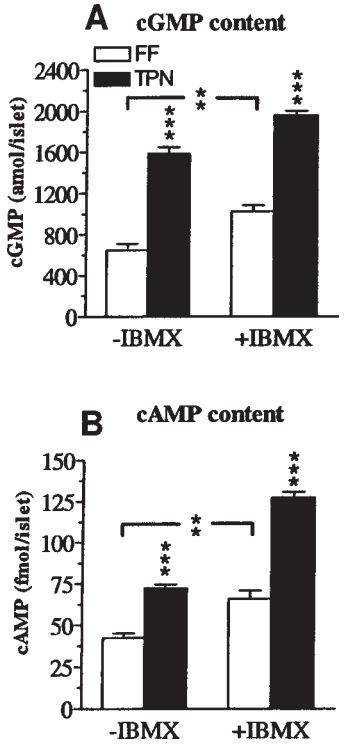

Fig. 3. Content of (**A**) cGMP and (**B**) cAMP in isolated islets from FF controls and TPN-treated rats in absence and presence of IBMX (0.20 mmol/L). Data are means ± SEM for four to six batches of islets taken from four to six animals in each group. Each batch was isolated from a single rat. Asterisks denote probability level of random difference for islets from FF vs TPN-treated rats and for differences in the absence and presence of IBMX. $^{**}p < 0.01$; $^{***}p < 0.001$.

of cAMP and the cyclic 5'-guanosine monophosphate (cGMP) in our TPN islets. Islets isolated from TPN-treated rats showed 130 and 90% higher content of cGMP and cAMP, respectively, than islets from their FF controls. Thus, cGMP was increased from 695 ± 64 amol/islet (FF controls) to 1586 ± 40 (TPN), and cAMP from 42.6 ± 2.6 fmol/islet (FF controls) to 80.4 ± 2.1 (TPN) ($p < 0.001$ for both) (Fig. 3A,B). To ensure that these differences did not emerge during the procedure of islet isolation, a series of experiments was conducted in which the islets were isolated in the continuous presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), a well-known inhibitor of cyclic nucleotide degradation. Higher levels of cGMP and cAMP were now found in both islets isolated from TPN-treated and from FF control animals. Therefore, cGMP was increased from 1586 ± 40 (–IBMX) to 1962 ± 40 amol/islet (+IBMX) ($p < 0.001$) and cAMP from 80.4 ± 2.1 (–IBMX) to 127.5 ± 3.5 fmol/islet (+IBMX) ($p < 0.001$) in TPN rats. In FF control islets, cGMP was increased from 695 ± 64 (–IBMX) to 1026 ± 62 (+IBMX) ($p < 0.001$) and cAMP from 42.6 ± 2.6 (–IBMX) to 66.0 ± 4.8 (+IBMX) ($p < 0.001$). However, it was evident that the relative increase in islet cAMP in TPN-treated as compared with FF control animals was approximately the same (150%), irrespective of whether the islets were isolated in the absence or presence of IBMX, whereas the relative increase in cGMP content was less pronounced in TPN islets (125%) as compared with control islets (150%) (Fig. 3).

Effect of TPN Treatment on IBMX-Stimulated Insulin and Glucagon Secretion from Isolated Islets

The secretory response of insulin to the phosphodiesterase inhibitor IBMX in the presence of a substimulatory concentration of glucose (4.0 mmol/L) was much greater from incubated islets isolated from TPN-treated rats compared with islets from FF controls (Fig. 4A). In this series of experiments, we used 4.0 mmol/L of glucose instead of 1.0 mmol/L since the insulin-releasing action of IBMX at 1.0 mmol/L of glucose is very poor (unpublished results). No appreciable differences in basal insulin secretion between the two groups

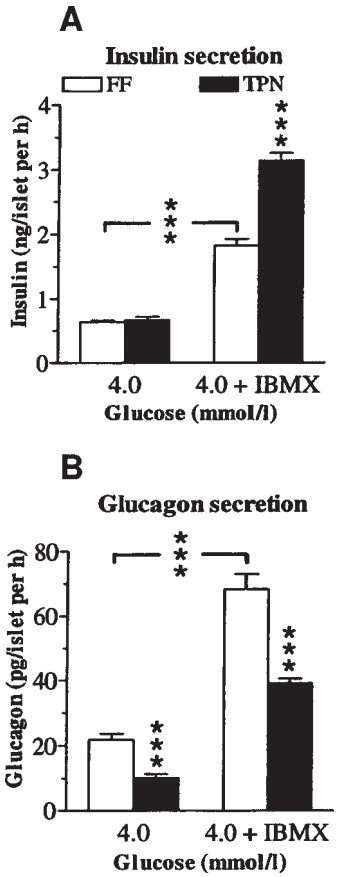

Fig. 4. (**A**) Insulin and (**B**) glucagon secretion from islets isolated from FF controls and TPN-treated rats and incubated for 60 min in basal glucose (4 mmol/L) in absence and presence of IBMX (1 mmol/L). Values are the means ± SEM for 10–12 batches of islets in each group. Each batch was isolated from a single rat. Asterisks denote probability level of random difference for islets from FF vs TPN-treated rats and for differences between 4.0 mmol/L of glucose in the absence and presence of IBMX. ***$p < 0.01$.

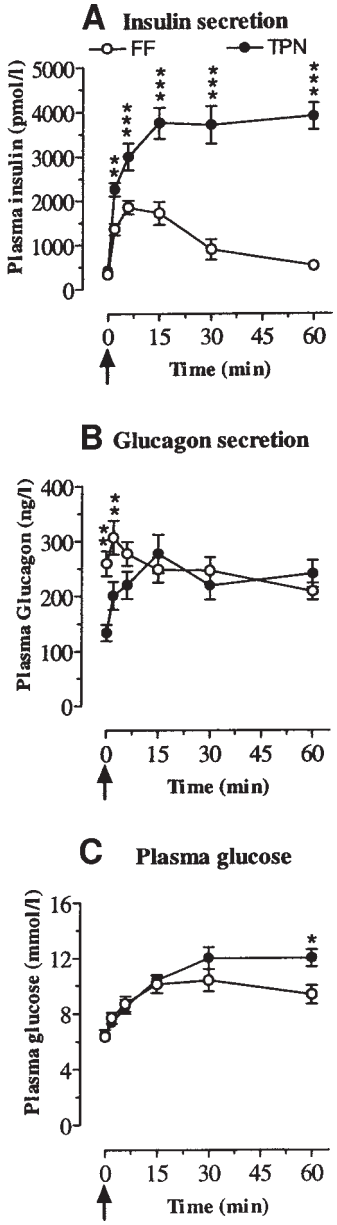

Fig. 5. Effect of an iv IBMX injection, 13.5 µmol/kg, on dynamics of (**A**) insulin and (**B**) glucagon secretion as well as (**C**) plasma glucose levels in FF controls and TPN-treated rats. The experiments were performed in unanesthetized rats kept in Bollman cages and supplied with a catheter in the right jugular vein. Arrows denote injection of IBMX at time 0. Values are the means ± SEM for 9 to 10 rats in each group. Asterisks denote the difference between FF and TPN-treated rats. *$p < 0.01$; **$p < 0.01$; ***$p < 0.001$.

could be seen in the presence of 4 mmol/L of glucose (Fig. 4A). Basal glucagon secretion, however, was lower from islets of TPN-treated animals (Fig. 4B). Moreover, in contrast to IBMX-stimulated insulin secretion, IBMX-stimulated glucagon secretion was lower from islets of TPN-treated rats than from controls (Fig. 4B). Note also that glucagon release from control islets was decreased at 4.0 mmol/L of glucose (Fig. 4B) compared with 1.0 mmol/L of glucose (Fig. 1B). This is in accordance with previous data *(15)* showing that glucose-induced suppression of glucagon release starts at very low levels of glucose.

Effect of iv Administration of IBMX on Plasma Levels of Insulin, Glucagon, and Glucose in TPN-Infused Rats

The next series of experiments examined the in vivo action of IBMX. After 10 d of TPN treatment, rats received a rapid iv injection of IBMX (13.5 µmol/kg), and the dynamics of the insulin, glucagon, and glucose responses were recorded and compared with the responses in an appropriate group of FF control animals. Figure 5 illustrates that the insulin secretory response of TPN-treated animals to IBMX was markedly enhanced compared with that of FF controls. The basal plasma insulin levels (at time 0) were similar in both groups. The basal plasma glucagon levels, however, were much lower in the TPN rats than in the FF controls. Injection of IBMX brought about a rapid increase in the glucagon levels in the TPN rats, reaching the same concentration as in the FF controls. Plasma glucose rose in both groups

of rats but was slightly higher in the TPN rats at the end of the time period studied.

Discussion

The present data show that when a TPN solution designed for rat purposes with the same components as used in clinical practice, is infused into normal Sprague-Dawley rats for 10 d, a strong iNOS expression and activity in their islets concomitant with a decrease in cNOS activity is induced. This is in agreement with our recent observation in mice subjected to chronic treatment with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Islets isolated from such mice showed that a strong increase in iNOS activity is accompanied by a concomitant decrease in cNOS activity, suggesting interactive mechanisms between islet iNOS and cNOS *(18)* in which iNOS-derived NO might exert a negative feedback on cNOS activity *(2)*. Furthermore, our TPN islets displayed a markedly reduced insulin response to glucose but an enhanced insulin secretion in the basal state, thus showing a certain similarity with earlier data from normal rat islets cultured with free fatty acids (FFA) *(22,23)*. The present results are therefore in agreement with previous reports from our laboratory *(8,10,12, 15,18)* showing that NO is an important negative modulator of glucose-stimulated insulin release. Longtime elevations of plasma lipids have previously been shown to interfere with islet glucose metabolism and hence have been assumed to contribute to an impairment of β-cell function *(22,23)*. Our present data show that an increased NO production could well be implicated in such an impairment of the insulin secretory process. Such a hypothesis is supported by a recent report *(27)* showing that culturing islets from the Zucker diabetic (fa/fa) rat in the presence of elevated FFA was associated with an overabundance of islet fat, upregulation of islet iNOS mRNA, and enhanced islet NO production and apoptosis. Hence, culture of diabetic islets in the presence of FFA *(28)* as well as in vivo infusion of lipids (via TPN) in normal healthy rats (present study) seem to upregulate islet iNOS expression and activity.

Insulin Secretion

In a recent study *(18)*, we found that chronic treatment of mice with the NOS inhibitor L-NAME paradoxically stimulated islet iNOS expression and activity. Islets isolated from such mice displayed an upregulation of the cAMP system to compensate for the inhibitory action of NO on glucose-stimulated insulin release.

Similar compensatory mechanisms by the cAMP system are suggested to be operating after lipid-induced NO production in our TPN islets. First, we observed that the content of both cAMP and cGMP was markedly enhanced in islets assayed directly after isolation from TPN-treated rats vs FF controls, whether or not the phosphodiesterase inhibitor IBMX was present during the isolation procedure. Second, we found that TPN islets incubated in the presence of IBMX released much more insulin than the control islets. Similarly, the insulin secretory response to IBMX in vivo was strongly increased in the TPN rats vs the controls. Furthermore, an enhanced and improved function of the cAMP pathway is suggested by the sustained elevation of the plasma insulin levels in the IBMX-injected TPN rats. An enhanced sensitivity and activity of the cAMP system also in the liver of the TPN rats might explain why the plasma glucose levels in these rats were slightly more increased than in FF control rats, since increases in cAMP stimulate both glycogenolysis and gluconeogenesis in the liver *(29)*. It has also been previously reported that an increase in cAMP in peripheral tissues is related to an inhibition of glucose uptake *(30)*, which, at least partly, might explain the hyperglycemia.

NO is known to increase soluble guanylate cyclase activity and cGMP production *(1,2)*. In accordance with the observed increase in iNOS activity in the islets of TPN-infused rats, cGMP levels were markedly enhanced. The accompanying increase in cAMP levels, on the other hand, might possibly be attributed to the cGMP-inhibited cAMP phosphodiesterase *(31)*. Hence, it cannot be excluded that this increase in islet cGMP content, which indirectly upregulates the cAMP system, might also contribute to the enhanced insulin secretion in incubated islets in the presence of IBMX. In the presence of high glucose, however, as documented in Fig. 1A, the increased cAMP levels were apparently insufficient to compensate fully for the inhibitory effect of NO on glucose-stimulated insulin release. In fact, this was not unexpected, since we have recently shown *(17)* that glucose itself increases islet NO production. We also recently found that there was a rather poor correlation between glucose-stimulated insulin release and the increase in islet cGMP production during different experimental conditions *(17)*; thus, it seems conceivable that cAMP and not cGMP is the most important insulin-releasing signal in the present context. Such an assumption is also supported, but not definitely proven, by previous data *(32,33)* showing that dibutyryl cGMP is not able to potentiate insulin release in isolated islets.

The mechanisms of the inhibitory effects of NO on glucose-stimulated insulin release are still unclear. An attractive hypothesis is that NO-induced formation of *S*-nitrosothiols *(32)* contributes to this inhibition by impairing important regulatory thiol groups, which for long have been known to be essential for glucose-stimulated insulin release *(28, 33)*. Thus, in this respect any direct or indirect modulatory effect of the cGMP system is probably overshadowed by the formation of impairing *S*-nitrosothiols. In this context, it should be noted that insulin secretagogues whose mechanisms of action are specifically directed at activating the cAMP system (e.g., β-adrenoceptor stimulation, forskolin stimulation, phosphodiesterase inhibition) seem to exert their insulin secretory effects independently of "critical"

thiol groups *(28,34)*. This is amply illustrated herein by the strong stimulating effect of IBMX on insulin release both in vitro and in vivo in spite of a high NO production in the TPN islets.

Glucagon Secretion

Regarding the pattern of glucagon secretion in TPN-infused rats, we found that the IBMX-activated glucagon release was reduced in vitro, but stimulated in vivo. Basal glucagon secretion, however, was clearly decreased both in vitro and in vivo. No effect of TPN infusion on glucagon release was recorded in vitro in the presence of high glucose. We originally proposed *(6)* that NO should be considered a novel physiologic stimulator of glucagon release, and we recently demonstrated that the glucagon-producing α-cells are richly equipped with the cNOS enzyme, whereas induction of iNOS enzyme was preferentially associated with insulin-producing β-cells and that only few scattered α-cells were found to display iNOS immunoreactivity *(7)*. Moreover, we have previously and repeatedly shown that glucagon release brought about by L-arginine or by cholinergic stimulation is suppressed by different types of NOS inhibitors both in vitro and in vivo *(6,10–12,14,16,18,36–38)*, suggesting that NO is a positive modulator of glucagon secretion. In addition, we have very recently found that NO gas stimulates glucagon release from incubated islets *(17)*. Hence, the present results seem to be difficult to reconcile with our previous data. However, glucose as well as insulin are powerful inhibitors of glucagon release and thus counteract the stimulating effect exerted by NO *(10,15)*.

Similar inhibitory effects are elicited by other nutrients such as α-ketoisocaproic acid *(12)*. Such a "nutrient" inhibition by the TPN lipids might possibly abolish the NO-stimulated glucagon release in the basal state (in vivo and in vitro) as well as during incubation in the presence of high glucose. On the other hand, an upregulation of the cAMP system seems to counteract the lipid-induced inhibition, as suggested by the improved and markedly increased glucagon response seen after in vivo injection of IBMX in the TPN rats. As already mentioned, the glucagon-producing α-cells do harbor the cNOS enzyme but are only capable of displaying negligible immunocytochemical traces of iNOS activity *(7)*. Hence, the large amount of iNOS-derived NO is probably confined to β-cells, may only marginally influence the peripherally located α-cells, and, if anything, may exert a negative feedback *(2)* on α-cell cNOS. In fact, most data suggest that α-cell cNOS activity is a major regulator of NO-stimulated glucagon release and that iNOS-derived NO has only a marginal role in this context *(6,10–12,14,16–18,34–36)*.

Summary

The present data suggest that the impairment of glucose-stimulated insulin release seen after long-term TPN in normal, healthy rats can be explained by the expression and activity of iNOS in the pancreatic islets. The NO-elicited dysfunction of the glucose-stimulated insulin release is apparently compensated for by a marked increase in the cAMP-dependent secretory pathway, which, in recent experiments in mice *(34)*, was found to operate independently of the NO system.

Materials and Methods

Drugs and Chemicals

Collagenase (CLS 4) was obtained from Worthington (Freehold, NJ). Bovine serum albumin was from ICN (High Wycombe, UK). L-Arginine, IBMX, EDTA, dithiothreitol (DTT), NADPH, and calmodulin were from Sigma (St. Louis, MO). The radioimmunoassay (RIA) kits for insulin and glucagon determination were obtained from Diagnostika (Falkenberg, Sweden) and Euro-Diagnostika (Malmö, Sweden), respectively.

Animals and Research Design

Male Sprague-Dawley rats (B&K, Sollentuna, Sweden) weighing 200–230 g were used in all experiments. The rats intended for TPN were intraperitoneally anesthetized with 5% chloral hydrate before operation. The operation was performed under sterile conditions. The neck of the rat was gently washed with iodine solution. A silicon rubber catheter, 0.037-in. od (Silastic; Dow Corning, Midland, MI), was inserted into the right jugular vein. The catheter was transferred to the skull subcutaneously and connected to a swivel via a protective coil attached to the skin of the skull. Immediately after surgery, all rats in the TPN group were infused with a 5% glucose solution at 2.0 mL/h for 12 h, followed by TPN at 300 mL/(kg·d). Every second day the catheters were flushed with 100 U/kg of low molecular weight heparin (Fragmin®; Pharmacia, Uppsala, Sweden). No oral intake, including water, was allowed during the infusion period. Control animals underwent the same operative procedure including insertion of a catheter, but no TPN infusion was performed. The catheters were similarly flushed with 100 U/kg of Fragmin every second day. The control animals were allowed free access to a standard pellet diet (B&K Universal) and tap water ad libitum. The TPN infusion experiments lasted 10 d. All animals were housed in metabolic cages with a constant temperature. A 12:12 h d and night cycle was provided. A detailed description of the methodology and the composition of the TPN solution (Table 1) has previously been reported *(24,26,39)*. The experiments were approved by the local animal welfare committee (Lund, Sweden).

Experimental Protocol

Preparation of isolated pancreatic islets from the rat was performed by retrograde injection of a collagenase solution via the bile pancreatic duct *(40)* directly after decapitation. Islets were then collected under a stereomicroscope at room temperature.

Table 1
Composition of Solution Used for TPN [a]

Component	Amount/1000 mL of final solution
Total nitrogen	2.6 g
Amino acids	16.6 g
Glucose	168 g
Glycerol	4.3 g
Soybean oil	39.2 g
Energy	1066 kcal (nitrogen energy not included)

[a]Drugs used were Vamin®, 40% glucose, 20% Intralipid®, Soluvit®, Vitalipid Adult®, and Tracel®. The solution also contained the following: electrolytes: 3678.4 mg of Na^+, 1563.9 mg of K^+, 27 mg of Ca^{2+}, 121.5 mg of Mg^{2+}, 7.84 µg of Cr^{2+}, 1.02 mg of Cu^{2+}, 0.86 mg of Fe^{2+}, 0.21 mg of Mn^{2+}, 5.10 mg of Zn^{2+}, 0.10 mg of I^-, 14.90 µg of Mo^{4+}, 25.10 mg of Se^{2-}; vitamins: 0.78 mg of A, 78.43 mg of C, 3.92 µg of D_2, 7.14 mg of E, 117.65 µg of K_1, 0.20 mg of B_1, 2.82 mg of B_2, 3.14 mg of B_6, 3.92 µg of B_{12}, 0.31 mg of folic acid, 31.37 mg of nicotine amid, 11.76 mg of pantothenic acid, 47.06 µg of biotin.

In Vitro Incubation

The freshly isolated islets were preincubated for 30 min at 37ºC in Krebs Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/L of HEPES, 0.1% bovine serum albumin, and 1.0 mmol/L of glucose as previously described *(10)*. Each incubation vial contained 12 islets in 1.0 mL of buffer solution and was gassed with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium supplemented with test agents, and the islets were incubated for 60 min. All incubations were performed at 37ºC in an incubation box (30 cycles/min). Immediately after incubation, aliquots of the medium were removed for assay of insulin and glucagon *(41–43)*.

Assay of Islet NOS Activities

For the assay of islet NOS activities, the freshly isolated islets were thoroughly washed and collected in ice-cold buffer (200 µL) containing 20 mmol/L of HEPES, 0.5 mmol/L of EDTA, and 1.0 mmol/L of DTT (pH 7.2) and stored at −20ºC for subsequent enzyme analysis. After sonication on ice, the buffer solution containing the islet homogenate was supplemented to contain also $CaCl_2$ (0.45 mmol/L), calmodulin (25 U/mL), NADPH (2.0 mmol/L), and L-arginine (0.2 mmol/L) in a total volume of 450 µL. For the assay of iNOS, both calmodulin and $CaCl_2$ were omitted from the assay buffer. The homogenates were then incubated at 37ºC under constant air bubbling (1.0 mL/min) as previously described *(10,18)*. Aliquots of the incubated medium (200 µL) were mixed with an equal volume of *o*-phtaldialdehyde reagent solution in a glass vial and then passed through a 1.0-mL Amprep CBA cation-exchange column for high-performance liquid chromatography analysis. The amount of L-citrulline formed (NO and L-citrulline are produced in equimolar concentrations) was then measured in a Hitachi F1000 fluorescence spectrophotometer (Merck, Darmstadt, Germany) as previously described *(10,18)*. Protein was determined according to the method of Bradford *(44)*.

Western Blot

Approximately 150 islets were collected in Hanks' buffer (100 µL) and sonicated on ice (three times for 10 s each). Homogenate samples representing 20 µg of total protein from islet tissue were then run on 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electrotransfer (10–15 V, 60 min) (semidry transfer cell; Bio-Rad, Richmond, CA). The membranes were blocked in 9 mmol/L of Tris-HCl (pH 7.4) containing 5% nonfat milk powder for 40 min at 37ºC. Immunoblotting with either rabbit antimouse ncNOS (N-7155) (1:2000) or rabbit antimouse iNOS (N-7782) (1:2000) (Sigma) was performed for 16 h at room temperature. The membrane was washed twice and then incubated with alkaline phosphatase–conjugated goat antirabbit IgG (1:10,000) (Sigma) for 90 min. Antibody binding to ncNOS and iNOS was detected using 0.25 mmol/L of CDP-Star™ (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminescence signal was visualized by exposing the membranes to Dupont Cronex® X-ray films for 1–5 min. The intensities of the bands were quantified by densitometry (Bio-Rad GS-710 Densitometer).

Determination of cGMP and cAMP

The methodology for the measurement of islet cGMP and cAMP has been described in detail previously *(8)*. Freshly isolated islets were collected in 0.5 mL of ice-cold 10% trichloroacetic acid (TCA) containing the phosphodiesterase inhibitor IBMX (0.2 mmol/L), followed by immediate freezing in a −70ºC ethanol bath. Before assay, 0.5 mL of H_2O was added, and the samples were sonicated for three times for 5 s each followed by centrifugation at 1100*g* for 15 min. The supernatants were collected and extracted with 4x2 mL of water-saturated diethyl ether. The aqueous phase was removed and freeze dried, using a Lyovac GT 2 freeze dryer. The residue was then dissolved in 450 µL of 50 mmol/L Na-acetate buffer (pH 6.2). The amounts of cGMP and cAMP were quantified with [^{125}I]-cGMP and [^{125}I]-cAMP RIA kits (RIANEN; DuPont, Boston, MA). [^{3}H]-cGMP was added to the TCA islet homogenate in order to determine the recovery of cAMP and cGMP during the ether extraction. The mean recovery was 90%.

In Vivo Experiments

The rats were kept conscious in Bollman cages. To avoid stress reaction at blood sampling, the tail roots of the rats were anesthetized with 1% lidocaine (Astra-Zeneca); 5 mm of the tail was cut with a sharp surgical blade; and a blood sample was taken from the tail to measure the basal levels of plasma insulin, glucagon, and glucose at time 0. Then

IBMX (13.5 µmol/kg of body wt) was injected as a bolus via the jugular catheter directly after stopping the TPN infusion. Blood samples were taken from the tail at 2, 6, 15, 30, and 60 min after IBMX injection. Plasma was collected; immediately frozen; and stored at –20ºC until analysis for insulin, glucagon, and glucose. The concentrations of insulin and glucagon in plasma were determined by RIA *(41–43)*. Plasma glucose concentrations were determined enzymatically *(45)*. The inter- and intraassay coefficients of variation were 6 and 3.5% for insulin; and 12.9 and 7.3% for glucagons, respectively. The lower limit of detection was 15 pmol/L for insulin and 15 ng/L for glucagon. The error for a double determination of glucose was ±3% with a lower limit of detection of 0.3 mmol/L.

Statistical Analyses

Levels of significance between sets of data were assessed using student's *t*-test for unpaired data or, where applicable, analysis of variance followed by Tukey-Kramer multiple comparisons test. Results are expressed as means ± SEM. Asterisks are used in Figs. 1–5 to denote probability levels of random differences for which $p < 0.05$ was considered significant.

Acknowledgments

We gratefully acknowledge the technical assistance of Britt-Marie Nilsson and the secretarial help of Björn Otterlin. This study was supported by the Swedish Medical Research Council (14X-4286), the Swedish Diabetes Association, the Albert Påhlsson Foundation, and the Åke Wiberg Foundation.

References

1. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). *Pharmacol. Rev.* **43,** 109–142.
2. Knowles, R. G. and Moncada, S. (1994). *Biochem. J.* **298,** 249–258.
3. Panagiotidis, G., Alm, P., and Lundquist, I. (1992). *Eur. J. Pharmacol.* **229,** 277–278.
4. Schmidt, H. H. H. W., Warner, T. D., Ishii, K., Sheng, H., and Murad, F. (1992). *Science* **255,** 721–723.
5. Corbett, J. A., Wang, J. L., Misko, T. P., Zhao, W., Hickey, W. F., and McDaniel, M. L. (1993). *Autoimmunity* **15,** 145–153.
6. Panagiotidis, G., Åkesson, B., Alm, P., and Lundquist, I. (1994). *Endocrine* **2,** 787–792.
7. Alm, P., Ekström, P., Henningsson, R., and Lundquist, I. (1999). *Diabetologia* **42,** 978–986.
8. Panagiotidis, G., Åkesson, B., Rydell, E., and Lundquist, I. (1995). *Br. J. Pharmacol.* **114,** 289–296.
9. Gross, R., Roye, M., Manteghetti, M., Hillaire-Buys, D., and Ribes, G. (1995). *Br. J. Pharmacol.* **116,** 1965–1972.
10. Salehi, A., Carlberg, M., Henningson, R., and Lundquist, I. (1996). *Am. J. Physiol.* **270,** C1634–C1641.
11. Åkesson, B., Mosén, H., Panagiotidis, G., and Lundquist, I. (1996). *Br. J. Pharmacol.* **119,** 758–764.
12. Salehi, A., Parandeh, F., and Lundquist, I. (1998). *Cell Signal.* **10,** 645–651.
13. Tsuura, Y., Ishida, H., Shinomura, T., Nishimura, M., and Seino, Y. (1998). *Biochem. Biophys. Res. Commun.* **252,** 34–38.
14. Henningsson, R. and Lundquist, I. (1998). *Am. J. Physiol.* **275,** E500–E506.
15. Åkesson, B., Henningsson, R., Salehi, A., and Lundquist, I. (1999). *J. Endocrinol.* **163,** 39–48.
16. Åkesson, B. and Lundquist, I. (1999). *J. Physiol.* **515,** 463–473.
17. Henningsson, R., Alm, P., Ekström, P., and Lundquist, I. (1999). *Diabetes* **48,** 66–76.
18. Henningsson, R., Alm, P., Lindström, E., and Lundquist, I. (2000). *Am. J. Physiol.* **279,** E95–E107.
19. Corbett, J. A. and McDaniel, M. L. (1992). *Diabetes* **41,** 897–903.
20. Eizirik, D. L. and Pavlovic, D. (1997). *Diabetes/Metab. Rev.* **13,** 293–307.
21. Mandrup-Poulsen, T. (1996). *Diabetologia* **39,** 1005–1029.
22. Unger, R. H. (1995). *Diabetes* **44,** 863–870.
23. Sako, Y. and Grill, V. E. (1990). *Endocrinology* **127,** 1580–1589.
24. Fan, B.-G., Salehi, A., Axelson, J., Sternby, B., Lundquist, I., Andrén-Sandberg, Å., and Ekelund, M. (1997). *Pancreas* **15,** 147–153.
25. Vigili de Kreutzenberg, S., Lisato, G., Riccio, A., Giunta, F., Bonato, R., Petolillo, M., Tiengo, A., and Del Prato, S. (1988). *Metabolism* **37,** 510–513.
26. Roth, B., Ekelund, M., Fan, B.-G., Hägerstrand, I., Salehi, A., Lundquist, I., and Nilsson-Ehle P. (1998). *Intensive Care Med.* **24,** 716–724.
27. Shimabukuro, M., Ohneda, M., Lee, Y., and Unger, R. H. (1997). *J. Clin. Invest.* **100,** 290–295.
28. Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 444–448.
29. Exton, J. H., Robinson, G. A., Sutherland, E. W., and Park, C. R. (1971). *J. Biol. Chem.* **246,** 6166–6177.
30. Bihler, J., Sarvh, P. C., and Sloan, J. G. (1978). *Biochim. Biophys. Acta* **510,** 349–360.
31. Trovati, M. and Anfossi, G. (1998). *Diabetologia* **41,** 609–622.
32. Lernmark, Å. (1974). *Horm. Res.* **5,** 227–233.
33. Jones, P. M., Persaud, S. J., Bjaaland, T., Pearson, J. D., and Howell, S. L. (1992). *Diabetologia* **35,** 1020–1027.
34. Hellman, B., Idahl, L.-Å., Lernmark, Å., Sehlin, J., and Täljedal, I.-B. (1974). *Excerpt. Med. Int. Congr. Ser.* **312,** 65–78.
35. Ammon, H. P. T. and Mark, M. (1985). *Cell Biochem. Funct.* **3,** 157–171.
36. Åkesson, B. and Lundquist, I. (1999). *Endocrine* **11,** 99–107.
37. Salehi, A., Parandeh, F., and Lundquist, I. (1998). *Biosci. Rep.* **18,** 19–28.
38. Åkesson, B. and Lundquist, I. (1998). *Biosci. Rep.* **18,** 199–213.
39. Salehi, A., Fan, B.-G., Ekelund, M., Nordin, G., and Lundquist, I. (2001). *Am. J. Physiol. (Endocrinol. Metab.)* **281,** E171–E179.
40. Gotoh, M., Maki, T., Kiyoizumi, T., Satomi, S., and Monaco, A. P. (1985). *Transplantation* **40,** 437,438.
41. Heding, L. (1966). In: *Labelled proteins in tracer studies.* Donato, L., Milhaud, G., and Sirchis, J. (eds.). Euratom: Brussels.
42. Ahrén, B. and Lundquist, I. (1982). *Diabetologia* **22,** 258–263.
43. Panagiotidis, G., Salehi, A., Westermark, P., and Lundquist, I. (1992). *Diabetes Res. Clin. Pract.* **18,** 167–171.
44. Bradford, M. M. A. (1978). *Anal. Biochem.* **84,** 309–312.
45. Bruss, M. and Black, A. L. (1978). *Anal. Biochem.* **84,** 309–312.