



LUND UNIVERSITY

Expression of islet iNOS and inhibition of glucose stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27.

Qader, Saleem; Jimenez, Javier; Ekelund, Mats; Lundquist, Ingmar; Salehi, Albert

Published in:

American Journal of Physiology: Endocrinology and Metabolism

DOI:

[10.1152/ajpendo.00172.2006](https://doi.org/10.1152/ajpendo.00172.2006)

2007

[Link to publication](#)

Citation for published version (APA):

Qader, S., Jimenez, J., Ekelund, M., Lundquist, I., & Salehi, A. (2007). Expression of islet iNOS and inhibition of glucose stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27. *American Journal of Physiology: Endocrinology and Metabolism*, 292(5), E1447-E1455.
<https://doi.org/10.1152/ajpendo.00172.2006>

Total number of authors:

5

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



LUND UNIVERSITY
Faculty of Medicine

LU:*research*

Institutional Repository of Lund University

This is an author produced version of a paper published in American journal of physiology. Endocrinology and metabolism. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Qader, Saleem S and Jimenez-Feltstrom, Javier and Ekelund, Mats and Lundquist, Ingmar and Salehi, Albert.

"Expression of islet iNOS and inhibition of glucose stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27 "

American journal of physiology. Endocrinology and metabolism, 2007, Issue: Jan 30.

<http://dx.doi.org/10.1152/ajpendo.00172.2006>

Access to the published version may
require journal subscription.

Published with permission from: American
Physiological Society

Expression of islet inducible nitric oxide synthase and inhibition of glucose stimulated insulin release after long-term lipid infusion in the rat is counteracted by PACAP27

Saleem S. Qader¹, Javier Jimenez-Feltström², Mats Ekelund¹, Ingmar Lundquist² and Albert Salehi²

Department of Surgery¹ and Institute of Physiological Sciences, Dept. Pharmacology²,
University of Lund, Sweden

Running title: Lipid infusion, islet iNOS and PACAP27

Key words: Lipid infusion, Pancreatic islets, Insulin secretion, isoforms of nitric oxide synthase, PACAP27,

Proofs and correspondence:

Dr. Albert Salehi

Institute of Physiological Sciences;

Dept. of Pharmacology; BMC F13

S-221 84 Lund, Sweden

E-mail: Salehi @farm.lu.se

Fax: +46 46 2224429

ABSTRACT

Chronic exposure of pancreatic islets to elevated plasma lipids such as free fatty acids (FFA) can lead to β -cell dysfunction which may over time become irreversible, a process referred to as lipotoxicity. The present investigation examined, by confocal microscopy and biochemical analysis, whether the expression of islet inducible nitric oxide synthase (iNOS) and the concomitant inhibition of glucose-stimulated insulin release after a sustained increased in plasma FFA due to lipid infusion in the rat could be modulated by the islet neuropeptide pituitary adenylate cyclase activating polypeptide 27 (PACAP27). After lipid infusion for 8 days freshly isolated islets showed a strong expression and activity of iNOS protein as detected by confocal microscopy and Western blot. The iNOS expression was still evident after incubation of the islets in the presence of 8.3 mmol/l glucose and was furthermore accompanied by a high iNOS-derived NO generation and a decreased insulin release concomitant with an increased cyclic GMP accumulation when compared to islets isolated from freely fed controls. No iNOS activity could be found in the islets of the control animals. Addition of PACAP27 to incubated islets from lipid-infused rats resulted in a great increase in islet cyclic AMP content, a decreased cyclic GMP content and a marked suppression of the activities of both neuronal constitutive NOS (ncNOS) and iNOS concomitant with an enhanced insulin response to glucose. These effects were reversed by the protein kinase A (PKA) inhibitor H-89. The results suggest that PACAP27 through its cyclic AMP and PKA stimulating capacity is able to strongly suppress not only ncNOS activity but most importantly also the lipid-induced increase of iNOS activity and hence to decrease the total production of NO and cyclic GMP in the islets. By doing so it restores the associated impairment of glucose-stimulated insulin release to normal levels and in addition it might have a cytoprotective effect against apoptosis and other deleterious actions of iNOS-derived NO in the β -cells.

INTRODUCTION

The solution used for total parenteral nutrition (TPN) contains a high amount of lipids which results in increased plasma levels of free fatty acids (FFA), triglycerides and cholesterol (8, 19, 22, 24). FFA has complex effects on pancreatic β -cells and insulin secretion (8, 9, 16, 19, 20, 22, 24, 30, 34). FFA acutely stimulates insulin secretion from isolated pancreatic islets (9) whereas long-term incubation (24 h) of islets in the presence of FFA negatively modulates β -cell function and results in a markedly decreased glucose-stimulated release of insulin (34). Indeed it has been known for a long time that chronic elevation of plasma FFA impairs the insulin secreting response to glucose both in humans and animals (8, 19, 20, 22, 24, 30, 34) and this hyperlipidemia has been suggested to be closely linked to type 2 diabetes (15). We have previously reported that nutrient-stimulated insulin secretion was markedly disrupted after lipid infusion in rats (8, 19, 22, 24) and that this insulin secretory defect could be reversed by agents stimulating cyclic AMP production (22, 23). We also found that islets of lipid-infused rats exhibited an increased nitric oxide (NO) production due to the induction of inducible NO synthase (iNOS) (22, 23).

In the present study we wanted to explore whether the cyclic AMP stimulating neuropeptide pituitary adenylate cyclase activating polypeptide 27 (PACAP27) would affect the increased expression and activity of iNOS as well as the impaired insulin response to glucose in islets isolated from lipid-infused rats. It should be recalled that PACAP, which originally was isolated from hypothalamus, is known to reside in islet parasympathetic nerve fibers and it has been found to be expressed also in islet β -cells (32, 33). It is a potent glucose-dependent stimulator of insulin release and exists in two main forms PACAP38 and PACAP27 (13). We have used the C-terminally truncated form i.e. PACAP27 which reportedly is equipotent to PACAP38 with respect to potentiation of glucose-stimulated insulin release (13, 32, 33). Because of its abundant localization within islet tissue PACAP has been regarded upon as an important regulator of insulin secretory processes, but detailed information on its mechanisms of action is still unclear (13, 32, 33).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (B&K, Sollentuna, Sweden) weighing 200-225 g were used in all experiments. The rats intended for lipid infusion were anesthetized

intraperitoneally with 5 % chloral hydrate before operation. The neck of the rat was shaved and the operative field washed with iodine solution. The operation was performed under sterile conditions. A silicon-rubber catheter, was inserted into the right external jugular vein. The catheter was delivered to the skull subcutaneously and connected to a swivel via a protective coil attached to the skin of skull. When the lipid-based solution composed for total parenteral nutrition (TPN) was infused the catheter was flushed with 100 U/kg/day of low molecular weight heparin (Fragmin; Pharmacia Corp., Uppsala, Sweden) every second day. The rats serving as freely fed controls underwent the same operative procedure, but no catheter was inserted. A detailed description of the methodology used and the composition of the lipid-based (Intralipid® Pharmacia) TPN solution have previously been reported (8, 22-24). The animals were infused for 8 days and there was no difference in body weights of control and lipid-infused rats at the end of the experiments. Further, as recently shown (24), blood glucose levels were within the normal range and the basal plasma insulin levels modestly enhanced after 8 days of lipid infusion. The concentrations of FFA, triglycerides and cholesterol in serum, however, were greatly increased by approximately 150, 80 and 90 %, respectively (24).

Chemicals. Bovine serum albumin was from ICN Biochemicals, High Wycombe, UK. PACAP27 was from Peninsula Europe (Merseyside, St. Helens, UK). The radioimmunoassay kits for cyclic AMP and cyclic GMP measurements were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The radioimmunoassay kits for insulin determination was obtained from Diagnostika (Falkenberg, Sweden). All other drugs and chemicals were from Sigma Chemicals, St Louis, MO or Marck AG, Darmstadt, Germany.

Isolation of pancreatic islets. Preparation of isolated pancreatic islets from the rat was performed with the Gotoh method; *i.e.* retrograde injection of a collagenase solution via the bile-pancreatic duct as previously described (21). Islets were collected under a stereomicroscope at room temperature, thoroughly washed, and then used immediately for the different experiments.

In vitro experiments. The freshly isolated islets were preincubated for 30 min at 37° C in Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1% bovine serum albumin, and 1.0 mmol/l glucose as previously described (11, 21). Each incubation vial contained 50 islets in 1.5 ml 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 supplemented with test agents, and the islets were incubated for 60 minutes. All incubations were performed at 37° C in an incubation box (30 cycles/min). Immediately after incubation, aliquots of the medium were removed for assay of insulin (21).

Confocal microscopy. The freshly isolated islets as well as incubated islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories, Inc, CA). iNOS were detected with the corresponding rabbit-raised primary antibody (BD Transduction Lab, CA) in combination with Cy2-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc). For staining of insulin, islets were incubated with a guinea pig-raised anti-insulin antibody (Eurodiagnostica, Malmö Sweden) followed by a Cy5-conjugated anti-guinea pig IgG antibody (Jackson Immunoresearch Laboratories, Inc). Fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 488/505-530nm (Cy2) and 633/>650nm (Cy5).

Assay of islet NOS activities. Preincubation and incubation of freshly isolated islets were performed as stated above with the exception that each incubation vial contained 200 islets in 1.5 ml of buffer solution and was incubated for 60 min. After incubation aliquots were removed for insulin determination and the islets were then thoroughly washed and collected in 200 µl buffer solution containing 20 mmol/l HEPES, 0.5 mmol/l EDTA and 1 mmol/l DL-dithiothreitol, and immediately frozen at -20°C. On the day of assay, the islets were sonicated on ice and for ncNOS activity the buffer solution was supplemented to contain also 0.45 mmol/l CaCl₂, 2 mmol/l NADPH, 25 U/ml calmodulin, and 0.2 mmol/l L-arginine. For the determination of iNOS activity both Ca²⁺ and calmodulin were omitted. The homogenate was then incubated at 37°C under constant air bubbling, 1.0 ml/min for 2 h. Aliquots of the incubated homogenate (200 µl) were then passed through an 1ml Amprep CBA cation-exchange column for high performance liquid chromatography (HPLC) analysis of the L-citrulline formed. The methodology has previously been described in detail (11, 21). Since L-citrulline is created in equimolar concentrations to NO, and since L-citrulline is stable whereas NO is not, L-citrulline is the preferred parameter when measuring NO production. Protein was determined according to Bradford on samples from the original homogenate (4).

Western blot analysis. Approximately 250 islets (n= 4 in each group) were collected in Hanks' buffer (100 µl) and sonicated on ice (3x10 s). Homogenate samples representing 10 µg of total protein from islet tissue were then run on 10 % SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electrotransfer (10-15 V, 60 min) (semi-dry transfer cell, B10-RAD, Richmond, CA). The membranes were blocked in 9 mmol/l Tris-HCl (pH 7.4) containing 5 % non-fat milk powder for 40 min at 37° C. Immunoblotting with rabbit anti-mouse iNOS (N-7782) (Sigma, St. Louis, MO) was performed for 16 h at room temperature. The membrane was washed twice and then incubated with alkaline-phosphatase conjugated goat anti-rabbit IgG (1: 10 000) (Sigma) for 90 min. Antibody binding to iNOS was detected using 0.25 mmol/l CDP-StarTM (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminiscence signal was visualized by exposing the membranes to Dupont Cronex® X-ray films for 1-5 min. An appropriate standard, *i.e.* molecular mass markers were run in all analyses. The intensities of the bands were quantified by densitometry (Bio-Rad GS-710 Densitometer).

Measurement of islet cyclic AMP and cyclic GMP. After incubation the islets were thoroughly washed in glucose-free KRB buffer and collected and stored in 500 µl of ice-cold 10 % trichloroacetic acid (TCA) containing the phosphodiesterase inhibitor IBMX (0.2 mmol/l), followed by immediate freezing in a -70° C ethanol bath. Before assay, 500 µl of H₂O was added, and the samples were sonicated (3x5 seconds) followed by centrifugation (1100x g) for 15 minutes. As previously described (7, 23) the supernatants were then collected and extracted with water-saturated diethyl ether (4x2 ml). The aqueous phase was removed and freeze dried, using a Lyovac GT 2 freeze dryer. The residue was then dissolved in 450 µl of Na-acetate buffer (50 mmol/l, pH 6.2). The amounts of cyclic AMP and cyclic GMP were quantified with [¹²⁵I]-cyclic AMP and [¹²⁵I]-cyclic GMP radioimmunoassay kits (RIANEN, Du Pont Company, Boston, MA). [³H] -cyclic GMP was added to the TCA islet homogenate in order to determine the recovery of cyclic AMP and cyclic GMP during the ether extraction. The mean recovery was 90 %.

Statistics. Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramers' multiple comparisons test. Results are expressed as means ± SEM.

RESULTS

Confocal microscopy and immunocytochemical findings. In the first experiment we wished to study the influence of long-term lipid infusion (8 days) on iNOS expression in the pancreatic islets. Insulin immunoreactivity is seen in Fig. 1A and D. As shown in Fig. 1B no iNOS immunoreactivity was observed in islets isolated from freely fed control rats, whereas after lipid infusion a majority of islet cells showed a strong immunoreactivity for iNOS (Fig. 1E). Double immunolabelling of islets from lipid-infused animals showed that most iNOS-immunoreactive cells also expressed insulin immunoreactivity (Fig. 1F). The immunocytochemical findings after lipid infusion were confirmed by Western blot analysis showing that there was a marked expression of iNOS protein in the islets (Fig. 1G).

We recently observed (23) that raising islet cyclic AMP levels brought about a marked suppression of the increased iNOS activity seen in islets exposed to high glucose (11, 22, 23). Hence the next series of experiments was designed to explore the effects of PACAP27 (a cyclic AMP stimulating agent) on the lipid-induced expression of islet iNOS after incubation in a modest, physiological concentration of glucose (8.3 mmol/l). The islets were isolated directly after stopping the lipid infusion. Figure 2 shows a confocal micrograph of the islets after incubation in the presence of 8.3 mmol/l glucose with or without addition of PACAP27 (100 nmol/l). As shown in Figure 2B and E no iNOS immunoreactivity was detected in control islets after the incubation at 8.3 mmol/l glucose either in the absence (B) or presence (E) of PACAP27. In contrast, the expression of iNOS (Fig. 2H) induced by lipid infusion was still evident in such islets after the incubation period. Addition of PACAP27 to the incubation media suppressed the expression of islet iNOS in lipid-infused rats (Fig. 2K). Double immunostaining of insulin and iNOS shows that insulin and iNOS immunoreactivities are confined to the same cells (Fig. 2I).

Influence of PACAP27 on ncNOS and iNOS activities and glucose-stimulated insulin secretion in incubated islets from control and lipid-infused rats. In the following experiments we studied the influence of PACAP27 in the presence of 8.3 mmol/l glucose on islet ncNOS and iNOS activities in relation to insulin release. Fig. 3A shows that PACAP27 (100 nmol/l) suppressed ncNOS activity in association with a concomitant potentiation of insulin release in islets taken from control rats and then incubated at 8.3 mmol/l glucose (Fig. 3C). No iNOS activity, however, could be detected in these control islets (Fig. 3A). As illustrated in Fig. 3B pancreatic islets taken from lipid-infused rats and then incubated in the presence of 8.3

mmol/l glucose showed a slight increase in ncNOS activity ($P<0.05$) and a marked increase in the activity of iNOS compared to islets from controls (Fig. 3A and 3B). Hence, the total NO production in the islets was greatly elevated after lipid infusion (compare total NOS in Fig 3A and 3B). The addition of PACAP27 to the incubated islets markedly suppressed the activity of ncNOS in control islets (Fig. 3A) and both ncNOS and iNOS and thus the total NOS activity in lipid-infused rats (Fig.3B). This suppressive effect of PACAP27 on iNOS and ncNOS activities was reversed when H-89 (an inhibitor of PKA), 2 $\mu\text{mol/l}$, was added to the incubation medium (Fig. 3B). In parallel to the suppressive effects of PACAP27 on islet iNOS and ncNOS activities in islets isolated after lipid infusion, the glucose-stimulated insulin secretion was markedly potentiated (Fig. 3C). Moreover, the suppressive effect of PACAP27 on NOS isoenzyme activities was totally reversed and even increased by addition of H-89 to the incubation medium (Fig. 3B). This was accompanied by a markedly reduced ability of PACAP27 to potentiate insulin secretion (Fig. 3C).

Effect of PACAP27 on islet cyclic AMP and cyclic GMP production in relation to glucose-stimulated insulin secretion in lipid-infused and control rats.

Since NO is known to have a stimulatory effect on cyclic GMP production, it was of interest to investigate the effect of PACAP27 on cyclic GMP levels in isolated islets taken from lipid-infused rats. The islets were thereby incubated in the presence of 8.3 mmol/l glucose for 60 min in the absence or presence of PACAP27 (100 nmol/l). As shown in Fig. 4A the insulin secretion was slightly lower ($p<0.05$), the cyclic AMP level slightly increased ($p<0.05$) (4B), while the cyclic GMP level was greatly elevated ($p<0.001$) (4C) in the presence of 8.3 mmol/l glucose in islets isolated from lipid-infused animals compared to the controls. Addition of PACAP27 to the incubation medium resulted in a marked suppression of cyclic GMP levels in the islets of lipid-infused rats. In contrast, PACAP27 did induce a much greater increase in cyclic AMP levels in islets of lipid-infused animals than in controls. The insulin secretory response to PACAP27 in islets taken from lipid-infused rats was also much greater compared to control islets (Fig. 4A).

Discussion

General

The metabolic syndrome and diabetes is often associated with an abnormal lipid metabolism (15, 16, 30, 34) and most type 2 diabetic patients exhibit an elevated plasma level of FFA in addition to enhanced plasma glucose concentrations (15). Although the pathogenesis of type 2

diabetes is multifactorial in origin, the pancreatic β -cell plays a crucial role in the development of the disease (15, 16, 30, 34), and chronic elevation of plasma levels of FFA has been attributed to β -cell dysfunction (15, 16, 30, 34).

Long term lipid infusion in rats is considered a good model to investigate the effect of increased plasma levels of lipids on β -cell function and insulin secretion (8, 19, 22, 24). We have previously shown that lipid-infused animals are characterized by hyperlipidemia (FFA, triglycerides and cholesterol), but at the same time they display normoglycemia (24) and there is no significant difference in blood glucose levels in these rats compared to freely fed control rats (24). From the present results we therefore suggest that in our rat model the increased NO production, exerted by a marked expression and activity of islet iNOS, and the associated reduction of the insulin secretory capacity of the β -cell is a consequence of increased plasma lipids and not due to glucose or a combination of elevated levels of both glucose and FFA (18) which might be still more deleterious (18). It should be emphasized that the present data do not exclude toxic effects of high concentrations of glucose alone on the β -cell function that, at least in part, might be exerted through the induction of iNOS (11).

By using immunocytochemistry and confocal microscopy, we confirmed our previously reported biochemical observations describing a high production of NO concomitant with a strong expression of iNOS and an increase in cyclic GMP in pancreatic islets isolated from lipid-infused rats (22, 23). The confocal micrographs revealed that the expression of iNOS was manifested throughout the entire islet, and hence, including most β -cells. This further indicates that the observed biological effects in the islets upon induction of iNOS by lipid infusion might be at least a contributing factor to the insulin secretory defect seen in these animals. Obviously cyclic GMP is not primarily a positive modulator of glucose-stimulated insulin release in lipid-infused rats, since an excessive increase in islet cyclic GMP content was associated with a significant reduction in insulin secretion. The slight increase in cyclic AMP content in the islets of lipid-infused rats was probably an effect of the cyclic GMP-inhibited cyclic AMP phosphodiesterase (27).

It is also well documented that destructive and apoptotic processes in pancreatic islets of animal models of type 1 diabetes are accompanied by induction of iNOS and a large production of NO (5, 6, 14). Such a reaction pattern although less pronounced and lacking any

signs of autoimmune processes and insulinitis was clearly evident in the islets isolated from lipid-infused rats and is most likely due to a signal/s produced by fat overloading during the long-standing lipid infusion. This is supported by previous observations showing that culturing islets isolated from the Zucker diabetic (fa/fa) rat in the presence of FFA was associated with an overabundance of islet fat and enhanced NO production and apoptosis (26).

In contrast to the consistent experimental support of a critical role of iNOS for degenerative and apoptotic processes in β -cells, significant controversy exists with regard to the role played by ncNOS in the regulation of insulin secretion (11, 22). A clear indication that ncNOS-derived NO deserves consideration as a negative modulator of glucose-stimulated insulin release is coming from the findings that inhibition of ncNOS by selective NOS inhibitors such as L-NAME or L-NMMA positively affects the insulin response to glucose (1, 10, 11, 17, 25, 29). Moreover, we have recently shown that stimulation of ncNOS-derived NO is inhibitory to first phase insulin release in perfused rat islets (11). The mechanisms of action of NO to restrain nutrient-stimulated insulin release are still unclear. We have previously hypothesized that S-nitrosylation of the glutathione system and/or important regulatory proteins at distal sites in the secretory process are possible targets (1, 11, 17, 25). In fact a new proteomic approach in brain tissue has revealed a wide range of metabolic and signaling proteins that might serve as targets for neuronally generated NO through S-nitrosylation processes (12). In contrast to the negative impact exerted by NO on nutrient-stimulated insulin release, this gaseous messenger seems to have no appreciable effect on insulin secretory mechanisms elicited by secretagogues directly activating the cyclic AMP system (2, 3, 21-23), since such secretagogues apparently act independently of regulating thiol-groups (3).

Effects of PACAP27

The ability of PACAP27 to potentiate glucose-stimulated insulin secretion by increasing cyclic AMP production in incubated islets isolated from control rats was accompanied by a marked suppression of ncNOS activity. Hence this is in good agreement with our previous data showing that an increased ncNOS activity may negatively affect the insulin response to glucose (11).

Quantitative biochemical analysis of iNOS and ncNOS activities in incubated islets of lipid-infused rats showed that PACAP27 dramatically reduced the activities of both isoenzymes as

well as the cyclic GMP production. Regarding iNOS, this effect was further supported by double immunostaining for insulin and iNOS showing a marked PACAP-induced reduction of the iNOS fluorescence intensity in the islets. The glucose-stimulated insulin secretory response of islets isolated from lipid-infused rats was strikingly enhanced by PACAP27 and even greater than in PACAP27-treated control islets confirming our previous data (23) showing that an increase in the β -cell cyclic AMP system elicited by addition of dibutyryl-cyclic AMP is compensatory to NO-induced impairment of nutrient-stimulated insulin release in islets from lipid-infused rats.

The cyclic AMP system is supposed to be the main messenger for PACAP to mediate insulin secretion from the β -cells (13) but recent observations (7) have shown that the action of cyclic AMP in the secretory process is not only elicited through activation of PKA but that also PKA-independent direct effects on distal steps in the stimulus-secretion coupling are operating. However, our present data speak in favor of PKA being of decisive importance for regulating islet NOS activities and insulin release in lipid-infused rats.

Concluding remarks

The present investigation reveals a novel pathway by which cyclic AMP, and hence PACAP27 may stimulate and reverse the reduced insulin response to glucose induced by long-term lipid infusion to rats. Specifically, we propose that the mechanism by which PACAP27 exerts this important stimulatory effect is by suppressing the expression and activity of iNOS through a cyclic AMP/PKA-dependent pathway. This proposal is supported by the findings that the suppression of iNOS activity in the islets isolated from lipid-infused rat was totally counteracted by a PKA selective inhibitor *i.e.* H-89. Moreover, the beneficial effect of PACAP27 in normalizing the defective insulin response to glucose in lipid-infused animals is further emphasized by the present finding that addition of PACAP27 to the incubation media augmented the insulin releasing capacity of islets from lipid-infused rats to values actually exceeding those found for islets from control animals. Apart from a role as a physiological regulator of insulin releasing mechanisms PACAP-agonism might be considered a potential therapy for type 2 diabetes since it was recently reported (28) that a rather specific agonist for the important VPAC 2 receptor on the β -cell could be used in the rat without appreciable side effects known to occur after systemic administration of PACAP itself (28). Such a PACAP agonist might also be useful and provide a rationale for attempting

to restore the reduced insulin secretion seen in patients undergoing TPN treatment (31) by activating the cyclic AMP/protein kinase A pathway in their β -cells.

Acknowledgements

This study was supported by the Swedish Medical Research Council (14X-4286), the Diabetes program (University of Lund), and the Magnus Bergvall, Albert Pahlsson and Crafoord Foundations. The technical assistance of Britt-Marie Nilsson is gratefully acknowledged.

Legends to Figures

Figure 1 (A-F). Confocal micrographs with immunostaining of islets taken “ex vivo” from control or lipid-infused rats. Control rats are illustrated in A-C and lipid-infused rats in D-F. A and D insulin immunoreactivity (red) E shows iNOS immunoreactivity (green). Co-localization of insulin/iNOS is seen as yellowish fluorescence (F). Bars indicate 20 μm . **(G)** Representative example of Western blot of islets taken from controls or lipid-infused rat and then incubated with iNOS antibody. The blots were performed with 20 μg of islet protein on each lane. Arrow indicates the molecular weight of 130 kDa (iNOS).

Figure 2. Confocal micrographs of rat islets. Islets isolated from controls or lipid-infused rat were incubated for 60 min in the presence of 8.3 mmol/l glucose (A-C controls and G-I lipid-infused) or glucose + PACAP27 (100 nmol/l) (D-F controls and J-L lipid-infused). After incubation the islets were double immunostained for insulin (red) and iNOS (green) and analyzed by confocal microscopy. Co-localization of insulin/iNOS appears as yellowish fluorescence (I). Bars indicate 20 μm .

Figure 3. Islet NO production from iNOS and ncNOS as well as total NO generation (Total NOS) measured as L-citrulline formation (A and B) and insulin release (C) from islets isolated from controls and lipid-infused rats, respectively, and then incubated at 8.3 mmol/l glucose in the absence or presence of PACAP27 (100 nmol/l) or PACAP27 + the PKA inhibitor H-89 (2 $\mu\text{mol/l}$). The means \pm SEM for 6-7 batches of islets per group from 6 independent experiments are shown. Asterisks (*) denote probability level of random difference vs islets incubated in the absence of PACAP27 or PACAP27 + H-89. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. Insulin secretion (A) and islet accumulation of cAMP (B) and cGMP (C) in the absence (open bars) or presence (black bars) of PACAP27 (100 nmol/l) in islets incubated with 8.3 mmol/ glucose. Islets were isolated from control rats or lipid-infused rats and then incubated for 60 min. The means \pm SEM for 6-8 batches of islets per group, isolated from 6 rats are shown. Asterisks (*) denote probability level of random difference. * $p < 0.05$; *** $p < 0.001$.

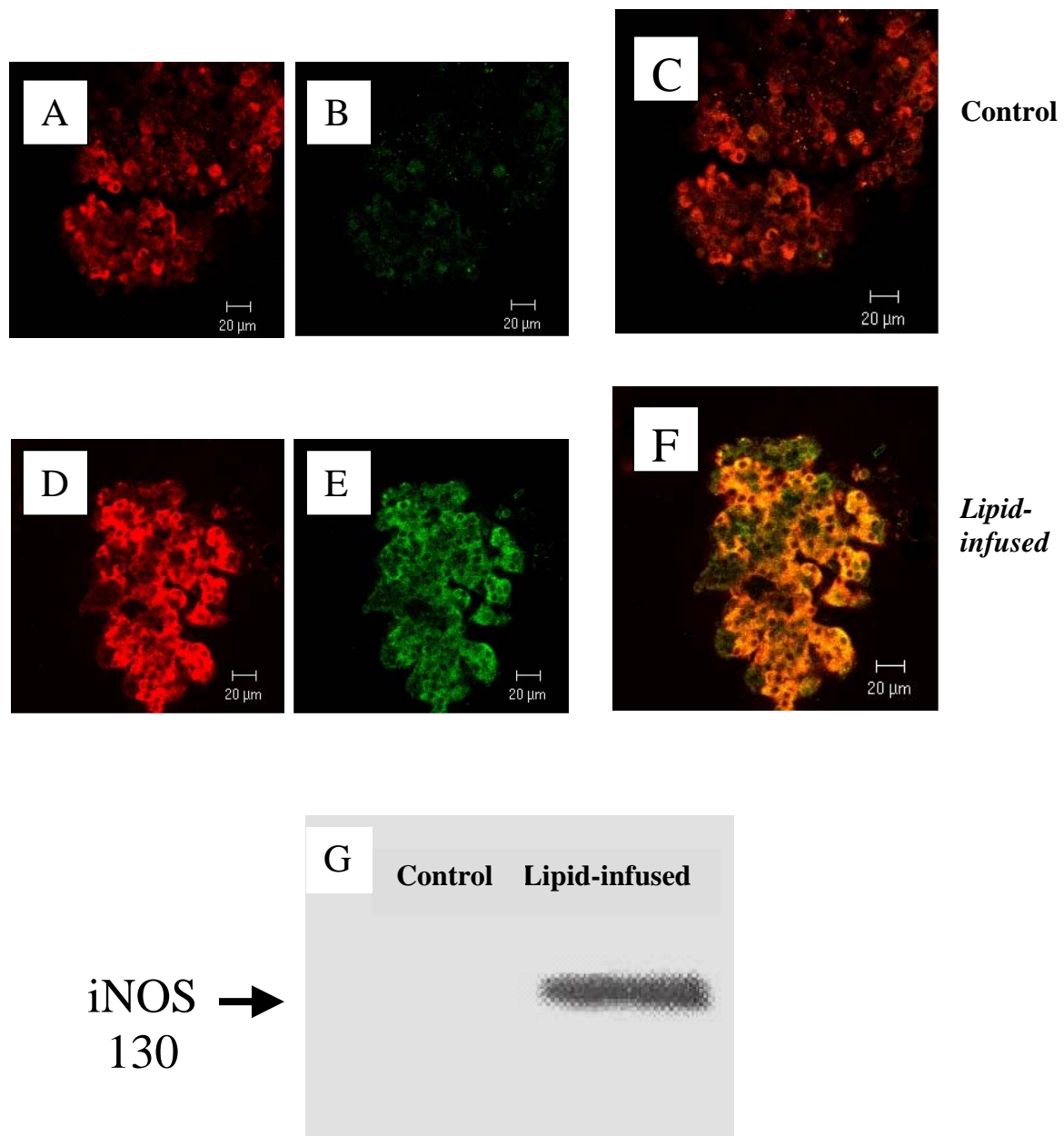


Fig-1

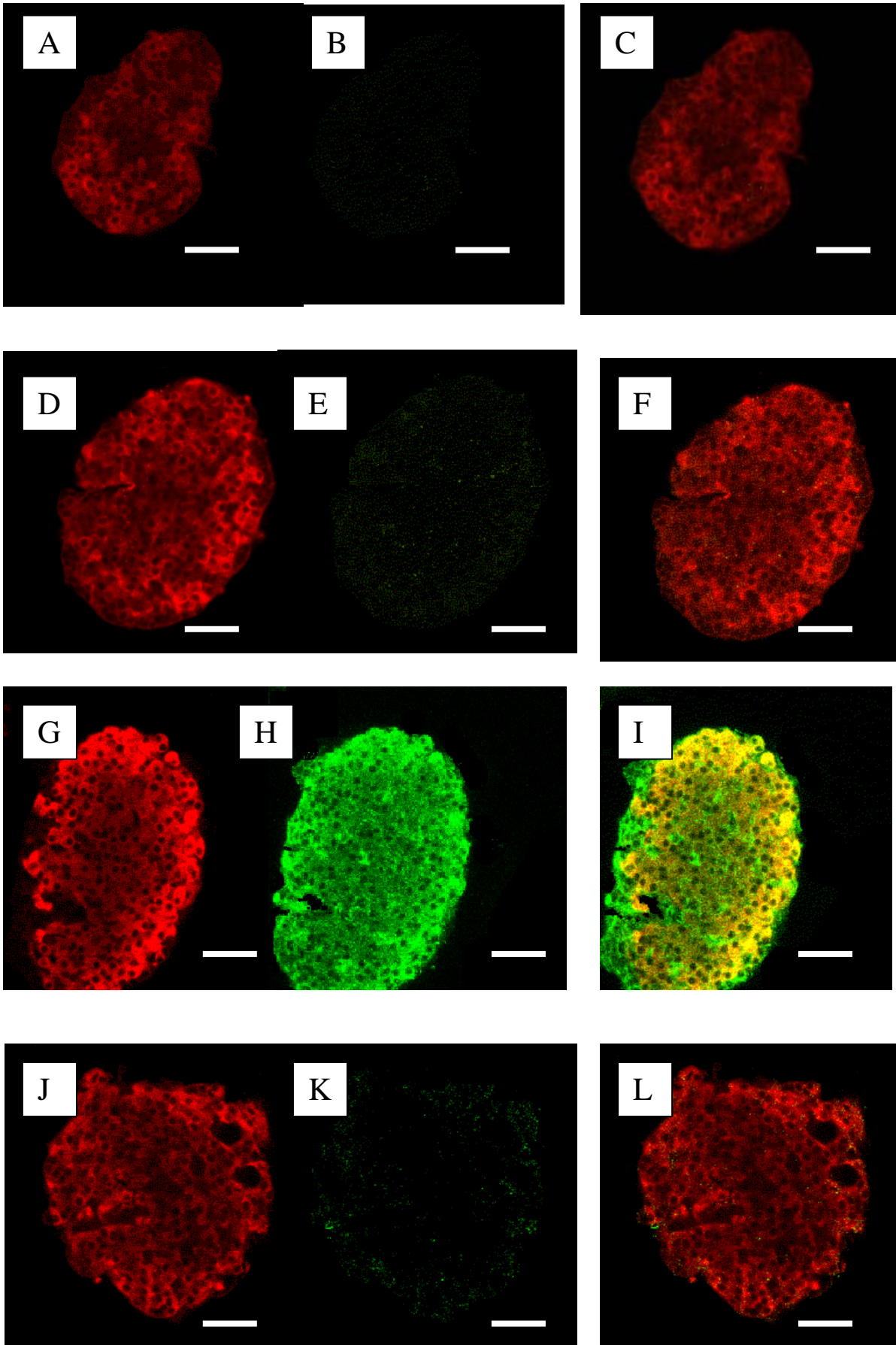


Fig. 2

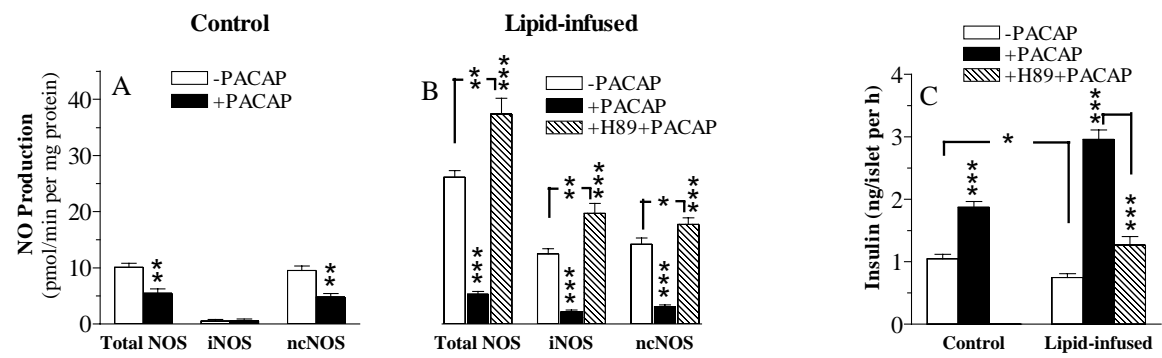


Fig. 3

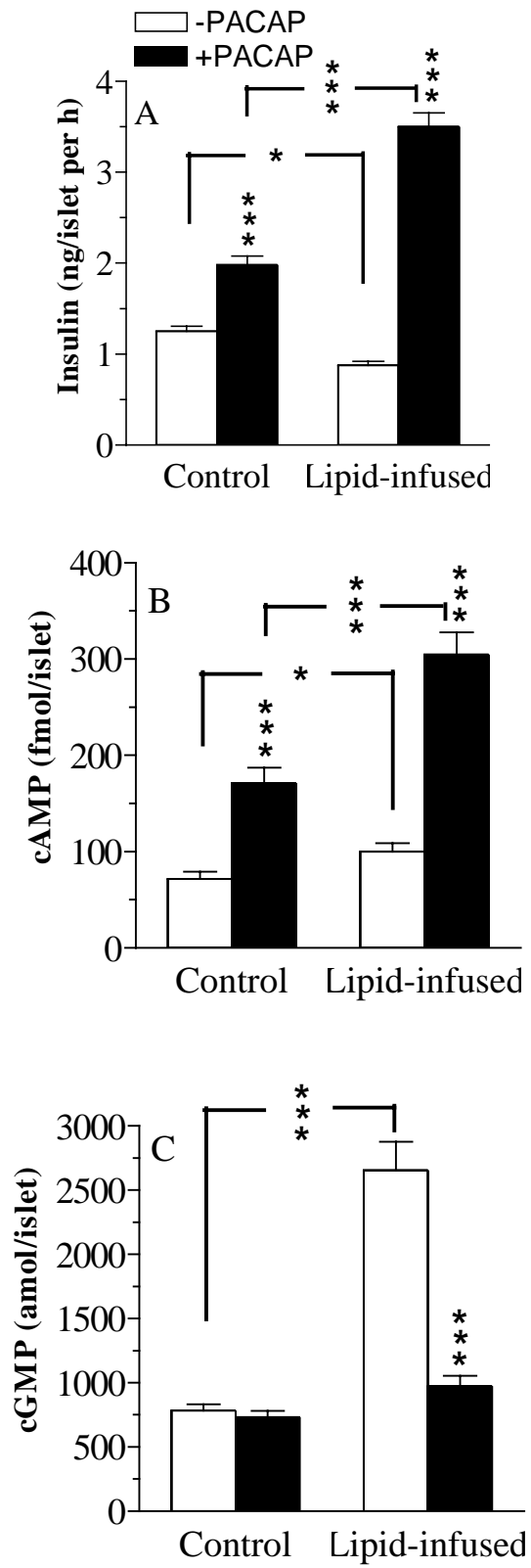


Fig. 4

References:

1. Akesson B, Henningsson R, Salehi A, and Lundquist I. Islet constitutive nitric oxide synthase and glucose regulation of insulin release in mice. *J Endocrinol* 163: 39-48, 1999.
2. Akesson B and Lundquist I. Nitric oxide and hydroperoxide affect islet hormone release and Ca(2+) efflux. *Endocrine* 11: 99-107, 1999.
3. Ammon HP and Mark M. Thiols and pancreatic beta-cell function: a review. *Cell Biochem Funct* 3: 157-171, 1985.
4. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
5. Corbett JA and McDaniel ML. Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. *Diabetes* 41: 897-903, 1992.
6. Eizirik DL, Flodstrom M, Karlens AE, and Welsh N. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* 39: 875-890, 1996.
7. Eliasson L, Ma X, Renstrom E, Barg S, Berggren PO, Galvanovskis J, Gromada J, Jing X, Lundquist I, Salehi A, Sewing S, and Rorsman P. SUR1 regulates PKA-independent cAMP-induced granule priming in mouse pancreatic B-cells. *J Gen Physiol* 121: 181-197, 2003.
8. Fan BG, Salehi A, Sternby B, Axelson J, Lundquist I, Andren-Sandberg A, and Ekelund M. Total parenteral nutrition influences both endocrine and exocrine function of rat pancreas. *Pancreas* 15: 147-153, 1997.
9. Felber JP and Vannotti A. Effects of Fat Infusion on Glucose Tolerance and Insulin Plasma Levels. *Med Exp Int J Exp Med* 10: 153-156, 1964.
10. Henningsson R, Alm P, Lindstrom E, and Lundquist I. Chronic blockade of NO synthase paradoxically increases islet NO production and modulates islet hormone release. *Am J Physiol Endocrinol Metab* 279: E95-E107, 2000.
11. Henningsson R, Salehi A, and Lundquist I. Role of nitric oxide synthase isoforms in glucose-stimulated insulin release. *Am J Physiol Cell Physiol* 283: C296-304, 2002.
12. Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, and Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193-197, 2001.
13. Jamen F, Puech R, Bockaert J, Brabet P, and Bertrand G. Pituitary adenylate cyclase-activating polypeptide receptors mediating insulin secretion in rodent pancreatic islets are coupled to adenylate cyclase but not to PLC. *Endocrinology* 143: 1253-1259, 2002.
14. Mandrup-Poulsen T. The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39: 1005-1029, 1996.
15. McGarry JD and Dobbins RL. Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* 42: 128-138, 1999.
16. Milburn JL, Jr., Hirose H, Lee YH, Nagasawa Y, Ogawa A, Ohneda M, BeltrandelRio H, Newgard CB, Johnson JH, and Unger RH. Pancreatic beta-cells in obesity. Evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. *J Biol Chem* 270: 1295-1299, 1995.
17. Panagiotidis G, Akesson B, Rydell EL, and Lundquist I. Influence of nitric oxide synthase inhibition, nitric oxide and hydroperoxide on insulin release induced by various secretagogues. *Br J Pharmacol* 114: 289-296, 1995.
18. Poitout V and Robertson RP. Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 143: 339-342, 2002.
19. Roth B, Ekelund M, Fan BG, Hagerstrand I, Salehi A, Lundquist I, and Nilsson-Ehle P. Biochemical and ultra-structural reactions to parenteral nutrition with two different fat emulsions in rats. *Intensive Care Med* 24: 716-724, 1998.
20. Sako Y and Grill VE. A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127: 1580-1589, 1990.
21. Salehi A, Carlberg M, Henningsson R, and Lundquist I. Islet constitutive nitric oxide synthase: biochemical determination and regulatory function. *Am J Physiol* 270: C1634-1641, 1996.

22. Salehi A, Ekelund M, Henningsson R, and Lundquist I. Total parenteral nutrition modulates hormone release by stimulating expression and activity of inducible nitric oxide synthase in rat pancreatic islets. *Endocrine* 16: 97-104, 2001.
23. Salehi A, Ekelund M, and Lundquist I. Total parenteral nutrition-stimulated activity of inducible nitric oxide synthase in rat pancreatic islets is suppressed by glucagon-like peptide-1. *Horm Metab Res* 35: 48-54, 2003.
24. Salehi A, Fan BG, Ekelund M, Nordin G, and Lundquist I. TPN-evoked dysfunction of islet lysosomal activity mediates impairment of glucose-stimulated insulin release. *Am J Physiol Endocrinol Metab* 281: E171-179, 2001.
25. Salehi A, Parandeh F, and Lundquist I. Signal transduction in islet hormone release: interaction of nitric oxide with basal and nutrient-induced hormone responses. *Cell Signal* 10: 645-651, 1998.
26. Shimabukuro M, Ohneda M, Lee Y, and Unger RH. Role of nitric oxide in obesity-induced beta cell disease. *J Clin Invest* 100: 290-295, 1997.
27. Trovati M and Anfossi G. Insulin, insulin resistance and platelet function: similarities with insulin effects on cultured vascular smooth muscle cells. *Diabetologia* 41: 609-622, 1998.
28. Tsutsumi M, Claus TH, Liang Y, Li Y, Yang L, Zhu J, Dela Cruz F, Peng X, Chen H, Yung SL, Hamren S, Livingston JN, and Pan CQ. A potent and highly selective VPAC2 agonist enhances glucose-induced insulin release and glucose disposal: a potential therapy for type 2 diabetes. *Diabetes* 51: 1453-1460, 2002.
29. Tsuura Y, Ishida H, Shinomura T, Nishimura M, and Seino Y. Endogenous nitric oxide inhibits glucose-induced insulin secretion by suppression of phosphofructokinase activity in pancreatic islets. *Biochem Biophys Res Commun* 252: 34-38, 1998.
30. Unger RH. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* 44: 863-870, 1995.
31. Vigili de Kreutzenberg S, Lisato G, Riccio A, Giunta F, Bonato R, Petolillo M, Tiengo A, and Del Prato S. Metabolic control during total parenteral nutrition: use of an artificial endocrine pancreas. *Metabolism* 37: 510-513, 1988.
32. Yada T, Sakurada M, Ihida K, Nakata M, Murata F, Arimura A, and Kikuchi M. Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet beta-cells. *J Biol Chem* 269: 1290-1293, 1994.
33. Yada T, Sakurada M, Ishihara H, Nakata M, Shioda S, Yaekura K, Hamakawa N, Yanagida K, Kikuchi M, and Oka Y. Pituitary adenylate cyclase-activating polypeptide (PACAP) is an islet substance serving as an intra-islet amplifier of glucose-induced insulin secretion in rats. *J Physiol* 505 (Pt 2): 319-328, 1997.
34. Zhou YP and Grill VE. Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J Clin Invest* 93: 870-876, 1994.