Streptozotocin (STZ) mediates acute upregulation of serum and pancreatic osteopontin (OPN): a novel islet-protective effect of OPN through inhibition of STZ-induced nitric oxide production

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Abstract

Osteopontin (OPN) is a secreted acidic phosphoprotein that binds to a cell-surface integrin-binding motif and is involved in many inflammatory and immune-modulating disorders. There is compelling evidence that soluble OPN can in a variety of situations help cells survive an otherwise lethal insult. In this study we show that OPN is localized in the rat pancreatic islets and ducts. Staining of pancreatic serial sections with islet hormone antibodies showed that all islet cells express OPN. Rats treated with a single dose of streptozotocin (STZ; 50 mg/kg) showed acute upregulation of serum OPN levels and pancreatic OPN mRNA and protein. Serum OPN dropped by the end of day 7 but was still higher than prediabetic levels. Pancreatic mRNA and protein showed a similar pattern. Twenty-four hours after STZ injection, the intensified OPN expression was localized towards the periphery of the islets and surrounded the remaining insulin-positive cells. To explore the significance of OPN acute upregulation, freshly isolated mildly diabetic islets (blood glucose <300 mg/dl) significantly improved their glucose-stimulated insulin secretion and reduced their NO levels. Next we investigated the regulation of OPN in β-cells. When STZ (5 mM) was added to the β-cell line RINm5F it significantly increased OPN mRNA levels within 6 h. To distinguish between the effect of STZ and high glucose on OPN transcription, RINm5F cells were transfected with luciferase-labeled rat OPN promoter and treated with STZ (0.05–5 mM) or with glucose (5–25 mM). STZ induced upregulation of OPN promoter activity within 3 h, while high glucose induced upregulation of OPN promoter activity after 48 h. Our data introduce OPN as a novel islet protein that is differentially regulated by STZ and glucose in the islets. OPN initial upregulation after diabetes induction was probably due to STZ-induced toxicity, while maintenance of the high OPN levels might be due to hyperglycemia. The acute induction of OPN after STZ-induced diabetes might represent an endogenous mechanism to protect the islets against STZ-induced cytotoxicity, partly via an RGD-dependent NO regulatory mechanism.


Introduction

Osteopontin (OPN) is an integrin- and calcium-binding multifunctional phosphoprotein produced by epithelial cells (Denhardt & Guo 1993), activated cells of the immune system (Patarca et al. 1993), cells of mineralized tissue (Denhardt et al. 2001), and bladder smooth muscle cells (Arafat et al. 2002). Overexpression of OPN has been reported in several physiological and pathological conditions, including immunologic disorders (Cantor 1995), neoplastic transformation (Senger et al. 1989), progression of metastasis (Craig et al. 1990), formation of urinary stones (Kohri et al. 1993), and wound healing (Liaw et al. 1998). Classical mediators of acute inflammation such as tumor necrosis factor α and interleukin-1β strongly induce OPN expression (Denhardt & Guo 1993, Patarca et al. 1993, Yu et al. 1999). OPN protein is selectively upregulated in the serum of type 1 diabetic patients (Fierabracci et al. 1999), in diabetic vascular walls (Takemoto et al. 2000a), and in diabetic kidneys (Fischer et al. 1998). Reports have shown that high glucose concentrations stimulate OPN expression in cultured smooth muscle cells (Takemoto et al. 2000b), partly through activation of high glucose and glucosamine-responsive elements in the OPN promoter (Asaumi et al. 2003).

β-Cell destructive insulitis in type I (Rabinovitch 1998) and streptozotocin (STZ)-induced experimental diabetes (Lukic et al. 1998, Rydgren & Sandler 2002) is associated

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with increased expression of proinflammatory cytokines, increased expression of the inducible NO synthase (iNOS) gene, and subsequent increased NO production. NO is hypothesized to deleteriously affect β-cell function by inducing apoptosis and suppressing glucose-stimulated insulin release (Corbett et al. 1993, Eizirik & Pavlovic 1997).

Many investigators have shown that both endogenous and exogenous OPN can inhibit induction of iNOS and have defined OPN as an important regulator of the NO signaling pathway (Singh et al. 1995, Rollo et al. 1996, Singh et al. 1999). It is not clear whether the reported OPN upregulation in diabetes is related to its role in regulation of NO production.

In the current study we examined the early temporal changes of serum OPN levels and the associated changes in its mRNA and protein expression in the pancreas in an experimental STZ–diabetic rat model. We investigated the functional significance of these changes in vitro using an STZ–induced cytotoxicity model. We also explored the regulation of OPN promoter by STZ and glucose in a rat β-cell line.

Research design and methods

Diabetes induction

All animal studies were performed in accordance with guidelines set forth by the Animal Care Committee of Thomas Jefferson University, Philadelphia, PA, USA. Adult male (200–250 g) Wistar rats (Harlan, Indianapolis, IN, USA; n=24) were injected intraperitoneally with a single dose of STZ (Biomol; 50 mg/kg body weight in 10 mM sodium citrate buffer, pH 4.5). Control animals (n=18) received only the vehicle buffer. Animals were allowed to free access to standard diet and water. Fasting blood from the tail vein was collected from animals prior to the experiment (day 0) and before death of the animals on days 1, 3, and 7 after STZ injection. Blood glucose was monitored using a glucometer (accu-check; Roche, Indianapolis, IN, USA). Animals were considered diabetic when fasting blood glucose was >250 mg/dl. Each experimental group comprised eight animals. Tissues from five experimental group comprised eight animals. Tissues from five different experimental groups were used for islet isolation and culture.

ELISA

Fasting OPN serum levels prior to induction of diabetes and in control and diabetic animals were measured using rat-specific ELISA kit (Assay Design, Ann Arbor, MI, USA). Spectrophotometric evaluation of OPN levels were made by Synergy HT multi-detection microplate reader (BioTeck, Winooski, VT, USA).

RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was isolated from pancreata of the different groups and from islets, using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Oligo (dT)15 (Promega)-primed cDNA was synthesized from 3-5 g total RNA using Moloney murine leukemia virus RT (Gibco BRL) at 37 °C for 60 min. Samples were incubated at 90 °C for 5 min to terminate the reverse transcription reaction. The cDNA mixtures (2 μl) were subjected to PCR using AmpliTag Gold DNA polymerase (PE Biosystems, Wellesley, MA, USA) and the following OPN primers were designed according to the published sequence of rat OPN cDNA (Oldberg et al. 1986): forward primer, 5’-AAGGCGCATTTAACGCAAAACACTCA-3’; reverse primer, 5’-CTCATCGAACCTGGGCTCTCAT-3’. iNOS primers were used to study the effect of OPN treatment of STZ–treated islets: forward primer, 5’-TCCGCGGACGCCTGTGAGACG-3’; reverse primer, 5’-GCTGGGGTGAGAGGGGTAGGTGATGT-3’. Upstream and downstream primers that could anneal with the 3’-untranslated region of rat GAPDH were included in the PCR reaction as an internal standard. GAPDH forward primer, 5’-GCA TGGCCTTCCGCTTTCTACC-3’; reverse primer, 5’-GGCCGCTGTCCACACCCTCTTCT-3’. The following conditions were used: 50 s at 94 °C, 90 s at 55 °C, and 150 s at 72 °C, with a 7-min final extension at 72 °C after 35 cycles. PCR products were electrophoresed on 2% agarose gels and band intensities were quantified using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

Sequence determination

PCR bands were purified from the agarose gel using the GeneClean II kit (BI 101, Carlsbad, CA, USA) according to the manufacturer’s protocol. Purified products were sequenced directly after estimating the concentration of DNA products. Sequences were aligned with published sequences using MegAlign sequence analysis software (DNASTAR, Madison, WI, USA) to confirm their identity.

Protein isolation and Western blot analysis

Pancreata from the different groups were lysed in modified RIPA lysis buffer (Gould & Hunter 1988), and the protein concentrations in the supernatant were determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal protein concentrations were loaded on 10% SDS/polyacrylamide slab gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA,
US). Blotted proteins were reacted with primary mouse monoclonal OPN antibody (1:150 diluted in Tween/PBS; (0-2% Tween in PBS, pH 7·4) Santa Cruz Biotechnology, Santa Cruz, CA, USA). Specificity of the antibody was evaluated by Western blot analysis with recombinant OPN protein containing a C-terminal His tag (Chemicon, Temecula, CA, USA). The protein bands were visualized with enhanced chemiluminescence reagents (ECL Plus Western Blotting Detection System; Amersham Biosciences), analyzed, and intensity-quantified using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

**Immunohistochemistry**

To localize OPN in the pancreas and study the changes in its expression after diabetes induction, formaline-fixed, paraffin-embedded tissue blocks were prepared from pancreata of the different groups. Serial sections at 5 µm were stained with the following antibodies: a monoclonal antibody against OPN (2A1; 1:100; Santa Cruz Biotechnology), ready-to-use antibodies against rat insulin, glucagon, and pancreatic polypeptide (BioGenex, La Jolla, CA, USA) and somatostatin (1:100; Accurate Chemical, Westbury, NY, USA). A vectastain universal elite ABC kit and diaminobenzidine chromogenic substrate (Vector Laboratories) were used according to the manufacturer’s protocol to visualize the tissue reaction to the antibodies. In the diabetic pancreata, double immunostaining was employed to visualize OPN and insulin in paraffin sections. Double immunostaining was also carried out in cryostat-cut 5 µm frozen sections to visualize OPN and infiltrating macrophages using antibody against CD68 (1:200; Dako, Carpinteria, CA, USA). Insulin and CD68 were visualized by alkaline phosphatase reaction (red), whereas diaminobenzidine was used to visualize OPN (brown).

**Islet isolation, culture, and STZ treatment**

Islets were isolated as mentioned elsewhere (Hill et al. 1999). Briefly, 20 ml cold Hank’s buffer/type IV collagenase solution was infused into the rat pancreatic parynchema after its dissection. The inflated pancreas was cleaned from the surrounding fat and lymph nodes, minced, and digested in a shaker-type water bath at 37 °C. Islets were recognized and handpicked under the stereo-microscope after their staining with dithiazone. Islets were aliquoted and cultured in RPMI medium containing 5 mM glucose and supplemented with 10 mM HEPES, 1% l-glutamine, and penicillin/streptomycin. Native rat OPN, a kind gift from Dr W. Butler, University of Texas, was used for these studies. Islets were allowed to equilibrate for 3 h before their treatment. Healthy islets with or without prior addition of OPN (0-15–15 nM) were treated with 0·5 mM STZ for 24 h after which islets and media were harvested. To evaluate whether OPN binding to the Arg-Gly-Asp (RGD) integrin-binding domain results in reduction of iNOS mRNA synthesis, cells were incubated with STZ+OPN (15 nM) +Gly-Arg-Gly-Asp-Asn-Pro (GRGDNP) peptide (1 mM), or with STZ+OPN (1·5 nM) +Gly-Arg-Ala-Asp-Ser-Pro (GRADSP; control) peptide (1 mM; Biomol, Plymouth Meeting, PA, USA). All concentrations were used according to our preliminary concentration studies with references to the values of nitrite release.

Diabetic islets were treated with OPN (0-15–15 nM) for 18 h after which glucose-stimulated insulin secretion (GSIS) studies and NO measurement were performed. We were able to isolate 800–1000 islets/pancreas from the vehicle-treated healthy animals, whereas from the diabetic animals (at day 7), and according to the severity of diabetes, we were able to isolate 0–200 islets/pancreas.

**NO determination**

In aqueous solution, NO is rapidly converted to nitrate and nitrite. The commercial kit we used (Calbiochem, La Jolla, CA, USA) includes a nitrate reductase step that converts nitrate to nitrite prior to quantitation using Griess reagent. Nitrite measurement was performed as an indirect measure of NO production. Spectrophotometric evaluation of nitrite levels was made by Synergy HT multi-detection microplate reader (BioTek).

**GSIS studies**

Immediately after islet isolation, 5–10 rat islets per experiment were cultured for 3 h in insert-containing 24-well plates (Corning, Corning, NY, USA) with 750 µl RPMI medium with 3 mM glucose, 10% fetal calf serum, 10 mM HEPES, 100 µ/l penicillin G, and 100 µg/ml streptomycin. Healthy islets were pretreated with OPN (0-15–15 nM) for 2 h before addition of STZ (0-5 mM). Diabetic islets were treated with OPN (0-15–15 nM) and maintained overnight at 37 °C. The next morning, the insert-containing islets were removed and transferred to RPMI medium with 3 mM glucose and incubated for 1 h, at which time the medium was sampled for insulin measurement. The medium glucose concentration was then increased to 17 mM and the islets incubated for an additional hour. Insulin assay was performed using rat-specific ultrasensitive insulin ELISA kit (DRG Diagnostics, Mountainside, NJ, USA).

**OPN promoter studies**

To explore the regulation of OPN in β-cells, RIN, clone 5F (RINm5F), an insulinoma cell line derived from the NEDH rat islet cell tumor, were used. Cells were purchased from American Type Culture Collection and grown at 37 °C under a humidified, 5% CO₂ atmosphere.
in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2·5 µg/ml amphotericin B. For gene-reporter assay, quiescent cells were obtained after 18 h incubation in serum-free medium. The rat OPN promoter (–1984 luc; GenBank accession number AF017274) in a luciferase expression vector pGL2 basic (Promega) was kindly provided by Dr S Mori, Chiba University, Japan (Takemoto et al. 2000, Asaumi et al. 2003). Cells were seeded into 24-well culture plates (10^5). At 80% confluence they were cotransfected with TransFast reagent (Promega), 0·5 µg pGL2 vectors containing the rat luciferase-labeled OPN promoter and 0·1 µg GFP as transfection control. Two hours later, serum-containing medium was overlaid and the cells incubated for an additional 24 h. The cells were then incubated with serum-free medium for 16 h followed by addition of STZ (0·05–5 mM) or different concentrations of glucose (5–25 mM). Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega) in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Transfection efficiency were normalized using the total protein concentration of the cell lysates.

**Statistical analysis**

All experiments were performed between four and six times. Data were analyzed for statistical significance by analysis of variance (ANOVA) with post-hoc Student’s t test analysis. These analyses were performed with the assistance of a computer program (JMP 5 software; SAS, Cary, NC, USA). Differences were considered significant at P ≤ 0·05.

**Results**

**OPN serum levels**

Temporal changes in serum OPN levels after diabetes induction was examined using a rat-specific ELISA kit. Serum OPN levels showed an initial 5-fold upregulation, 24 h after injection of STZ (day 1). Levels then came down but were still higher than prediabetic levels by the end of day 7. n = 8 in STZ-treated and n = 6 in vehicle-treated groups. *P < 0·05 versus control prediabetic values.

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Serum OPN levels. Serum levels of OPN were examined in STZ- and control vehicle-treated animals using rat-specific ELISA kit. Serum levels of OPN showed an initial 5-fold upregulation 24 h after injection of STZ (day 1). Levels then came down but were still higher than prediabetic levels by the end of day 7. n = 8 in STZ-treated and n = 6 in vehicle-treated groups. *P < 0·05 versus control prediabetic values.

**Expression of OPN protein and mRNA in control and diabetic pancreas**

Equal concentrations of the isolated protein from control and diabetic pancreata at the specified time points were electrophoresed using 10% SDS/PAGE. OPN protein molecular-mass isoforms were detected at ~65 and ~50 kDa. The 50 kDa band showed acute upregulation by day 1 (Fig. 2A), while the 65 kDa band showed early minimal changes. On days 3 and 7 the 50 kDa band was downregulated but was still significantly higher than the prediabetic levels.

Semiquantitative PCR of OPN mRNA and correction of the band intensity with GAPDH showed the same pattern seen with the protein (Fig. 2B). These data indicate that the pancreas might be an active source of serum OPN.

**OPN distribution in the control and diabetic pancreas**

Clear OPN immunoreactivity could be seen in vehicle-treated rats. OPN-reactive cells were seen in the islets and in the pancreatic ducts (Fig. 3B and D). Some islets showed more OPN reactivity than others. Staining of serial sections with islet hormone antibodies revealed the expression of OPN by other hormone-secreting cells (Fig. 3ii). Twenty-four hours after STZ injection, double immunostaining showed a more peripheral distribution of intensely stained OPN–reactive cells surrounding few insulin-positive cells (Fig. 3 iiiB). OPN-intensified reactivity at this early time point was not associated with infiltrating macrophages (Fig. 3 iiiA), indicating that this is a constitutive expression of OPN by islet cells and not by infiltrating macrophages that are reported to secret OPN (Masutani et al. 2003).

**OPN inhibits NO production in STZ-treated healthy islets**

Next we investigated the functional significance of the high levels of OPN in the islets. NO production was
measured in rat islets exposed to STZ (0.5 mM) plus or minus pretreatment with OPN (0.15–15 nM). Nitrite measurement was performed as an indirect measure of NO production in the media collected from the islets. The significantly elevated STZ-induced NO levels were dose-dependently reduced in the presence of OPN (Fig. 4A). Islets that were treated with OPN alone did not show significant changes when compared with untreated islets. OPN contains an RGD integrin-binding domain (Denhardt & Guo 1993, Guo et al. 2001). The hexapeptide GRGDSP, which blocks binding of OPN to cell-surface integrins (Singh et al. 1995), was used to determine the receptor-mediated effects of OPN. Cells were pretreated with GRGDSP hexapeptide (1 mM). In comparison to islets treated with GRADSP control peptide, GRGDSP (1 mM) was found to significantly inhibit the OPN-mediated decrease in iNOS synthesis (Fig. 4B), suggesting a requirement for OPN-integrin receptor binding for OPN-mediated NO regulation.

**OPN inhibits NO production and improves the GSIS in diabetic islets**

To test whether addition of OPN could benefit the diabetic islets, we isolated the islets from 1-week diabetic rats. Islets isolated from severely diabetic animals (blood glucose >400 mg/dl) produced low levels of NO and had defective GSIS. Addition of OPN to those islets did not significantly affect their nitrite production and did not improve their GSIS (data not shown). However, islets isolated from less severely diabetic animals (blood glucose <300 mg/dl) produced high levels of NO. Addition of OPN (0.15–15 nM) significantly reduced their NO levels (Fig. 5A).

Healthy rat islets showed a ∼10-fold increase in insulin secretion with 17 mM glucose, whereas diabetic islets showed β-cell dysfunction and significantly lower insulin surge at 17 mM. Diabetic islets showed a dose-dependent significant improvement in GSIS after treatment with OPN and a 40–60% restoration of control values (Fig. 5B), showing that OPN is capable of improving the islet function only in islets that are not severely dysfunctional.

**Induction of OPN mRNA by STZ in RINm5F β-cells**

To evaluate the expression of OPN in the RINm5F β-cell line and its regulation by STZ, we treated the cells with STZ (5 mM). OPN mRNA was induced only with the highest dose of STZ (5 mM) after 6 h (Fig. 6A). The concentration and duration of STZ treatment was determined by dose- and time-response studies (data not shown).

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Regulation of OPN promoter by STZ and glucose

Previous studies have shown that OPN promoter contains glucose-responsive elements (Asaumi et al. 2003). To investigate whether the induced rise of OPN levels is due to STZ or glucose, RINm5F cells were transfected with rat OPN promoter/luciferase gene construct. After 24 h of transfection, the cells were incubated with different concentrations of STZ (0–5 mM) and glucose (1–50 mM) for 3, 6, 24, and 48 h. Relative luciferase activity was calculated after deduction of the activity levels with pGL2 vector alone. High doses of STZ (5 mM) increased OPN promoter activity 1.5-fold after 3 h (Fig. 6B). Glucose induced a dose-dependent upregulation of OPN promoter activity after 48 h. These data show that the OPN promoter responds differentially, time-wise, to STZ and glucose.

Discussion

Whereas pathological changes within the pancreas, and in particular the islets, have been a major focus in diabetes, molecular changes in the islets and their microenvironment represent a major feature of diabetic islet injury. In this study, we introduce a new player, OPN, in the islet response to STZ-diabetes induction.

OPN is a highly hydrophilic and negatively charged sialoprotein of ~298 amino acids that contains a Gly–Arg–Gly–Asp–Ser (GRGDS) sequence. It is a secreted protein with diverse regulatory functions, including cell adhesion and migration, tumor growth and metastasis, atherosclerosis, aortic valve calcification, and repair of myocardial injury. Its expression is tissue specific and subject to regulation by many factors (Denhardt & Guo 1993, Patarca et al. 1993, Denhardt et al. 2001). Constitutive expression of OPN is found in bone (McKee & Nanci...
In vivo expression of OPN has previously been analyzed in several diabetic animal models in different tissues. Towler et al. (1998) demonstrated the upregulation of OPN expression in the aortas of high-fat diet-induced diabetic mice. Fischer et al. (1998) reported that the upregulation of OPN expression in the renal cortex of STZ-induced diabetic rats was mediated by bradykinin. Aspord et al. (2004) reported OPN among the genes specifically activated in the islets and lymph nodes in non-obese diabetic (NOD) mice. However, data concerning the early changes in OPN expression after diabetes induction and its functional significance have not been reported previously. In the present study, we demonstrate for the first time that as early as 24 h, OPN expression levels are enhanced in the serum and in diabetic pancreas, suggesting that diabetes-induced upregulation of OPN expression is a general phenomenon observed across the different tissues. We also show that OPN is constitutively expressed in the rat pancreatic islets and its expression is intensified after STZ-diabetes induction. However, when OPN serum levels were substantially reduced by day 7, the pancreas continued to produce OPN (Fig. 2A and B), suggesting an autocrine/paracrine role for endogenous pancreatic OPN. The enhanced OPN reactivity was not associated with accumulation of inflammatory cells in the islets (Fig. 3 iii), implicating islet OPN in the early islet response to STZ-induced cytotoxicity.

One of the important pathogenetic mechanisms of β-cell damage during experimental STZ-induced diabetes in rats and probably also in human insulin-dependent diabetes mellitus is the cytokine-induced overproduction of NO by iNOS, with subsequent increase of local oxidative stress in the pancreatic islets (Kwon et al. 1994, Haluzik & Nedvidkova 2000). We show here that addition of STZ to isolated islets induced a significant upregulation of NO levels (Fig. 4A). Interestingly, the same treatment induced significant upregulation of endogenous OPN mRNA and promoter activity (Fig. 6A and B). It would be of interest to know whether a more classical NO donor compound than STZ regulated OPN, and whether NO mediated the cytokine-induced increase in OPN expression. Studies addressing these questions are ongoing in our laboratory.

The relationship between NO and OPN has been examined by a number of investigators. Rollo et al. (1996) demonstrated that exogenous recombinant OPN protein

Figure 4 (A) OPN effect on STZ-induced nitrite formation in healthy islets. Healthy islets were pretreated with OPN (0.15–15 nM) for 2 h before the addition of STZ (0.5 mM) for an additional 24 h. Nitrite measurement was performed as an indirect measure of NO production in the media collected from the islets. Significant reduction in NO levels can be seen in islets treated with OPN when compared with STZ alone. OPN at high doses did not induce significant NO reduction. Data are expressed as means ± S.E.M. Each experiment was performed in duplicate and repeated three times for reproducibility. *P ≤ 0.05 versus control values and #P ≤ 0.05 versus STZ values, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student’s t test. (B) PCR analysis of iNOS and GAPDH mRNA transcripts from islets treated with STZ (0.5 mM; lane 2) plus or minus pretreatment with OPN (1.5 nM; lane 3) with prior addition of exogenous GRGDSP (1 nM; lane 4) or GRADSP (1 nM; lane 5). Addition of the GRGDSP peptide to the islet blocked OPN-mediated reduction of iNOS mRNA. 492 and 109 bp bands correspond to the amplified iNOS and GAPDH, respectively. Values are expressed as mean ± S.E.M. of three experiments. *P < 0.05 versus untreated islets; #P < 0.05 versus STZ-treated islets using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student’s t test.
was effective in blocking RAW264.7 murine macrophage NO production and cytotoxicity toward the NO-sensitive mastocytoma cells. Their work suggested that OPN in extracellular fluid might protect certain tumor cells from macrophage-mediated destruction by inhibiting the synthesis of NO. However, these authors did not attempt to localize a potential cellular source for OPN in this setting. Singh et al. (1999) reported that a synthetic 20-amino acid OPN peptide analogue decreased iNOS mRNA and protein levels in ventricular myocytes and cardiac microvascular endothelial cells. Transfection of cardiac microvascular endothelial cells with an antisense OPN cDNA increased iNOS mRNA in response to interleukin-1β and interferon-γ, suggesting that endogenous OPN inhibits NO production (Singh et al. 1995). Hwang et al. (1994) found that OPN suppressed NO synthesis induced by interferon and lipopolysaccharide in primary mouse kidney proximal tubule epithelial cells, suggesting a regulatory role for OPN in the NO signaling pathway. Data from our study clearly demonstrate that OPN inhibits the STZ-mediated induction of NO in the islets through downregulation of iNOS synthesis and suggest that OPN is an important regulator of the NO signaling pathway and NO-mediated cytoprotective processes in the islets. In addition OPN reduced the NO levels in mildly diabetic islets and improved their GSIS. However, NO levels were surprisingly low in the severely diabetic islets and were not affected by exogenous OPN treatment, indicating that OPN could be used to rescue islets that are not severely dysfunctional. High doses of OPN did not reduce STZ-derived nitrite or nitrate from STZ-diabetic islets in a dose-dependent manner, but did it dose-dependently restore the GSIS in the diabetic islets, suggesting the involvement of other mechanisms that mediate OPN protective effects.

Our data show that the anti-iNOS effect of OPN appears to be mediated by a membrane-bound integrin receptor because this effect was reversed when a peptide that blocks the integrin receptor was added (Fig. 4B). Nevertheless, a number of features of this OPN-NO regulatory system remain to be clarified and the specific signaling pathway by which OPN ultimately modulates iNOS synthesis in STZ-treated islets requires further studies. For example, it would be important to determine whether OPN plays a role in the regulation of nuclear factor-κB, which is a primary transcription factor necessary for iNOS synthesis (Flodstrom et al. 1996, Cardozo et al. 2001), and whether OPN regulates additional signaling cascades that are activated by STZ.

In this study, we also showed that OPN promoter was regulated by STZ and glucose. STZ induced an early (3 h) upregulation, while glucose induced a late (48 h) upregulation. This is suggestive that the early rise in pancreatic OPN after diabetes induction is a response to STZ-induced cytotoxicity, whereas the late rise is hyperglycemia-induced. It is not known, however, whether there are STZ specific cis-element on the OPN promoter and whether or not the STZ-induced OPN upregulation is mediated through NO, which has been reported to induce OPN transcription in macrophages (Guo et al. 2001). Studies into this question are currently ongoing in our laboratory.
Figure 6 (A) OPN mRNA expression in RINm5F cells. Cells were cultured for 6 h under control conditions (lane 1) or with STZ (5 mM; lane 2). The concentration and duration of STZ treatment was determined by dose- and time-response studies (data not shown). OPN and GAPDH mRNA content were analyzed by RT-PCR. The OPN mRNA contents are expressed as optical densities corrected for GAPDH. (468 and 109 bp bands correspond to the amplified OPN and GAPDH, respectively). Values are expressed as means ± S.E.M. from three experiments. *P<0.05 versus untreated cells using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student’s t test. (B) Induction of luciferase activity with STZ in RINm5F cells transfected with rat OPN promoter/luciferase gene construct. After 24 h of transfection, the cells were incubated with different concentrations of STZ, as shown, for 3 h. After incubation, the luciferase activity in the cell lysates was measured. STZ causes a dose-dependent increase in OPN promoter activity. Relative luciferase activity was calculated after deduction of the activity levels with pGL2 vector alone. Results represent means ± S.E.M from triplicate determinations. All experiments were repeated at least three times to confirm the reproducibility of the observations. (C) Induction of luciferase activity with glucose in RINm5F cells transfected with rat OPN promoter/luciferase gene construct. After 24 h of transfection, the cells were incubated with different concentrations of glucose, as shown, for 48 h. After incubation, the luciferase activity in the cell lysates was measured. Glucose induces a dose-dependent increase in OPN promoter activity. Relative luciferase activity was calculated after deduction of the activity levels with pGL2 vector alone. Results represent means ± S.E.M from triplicate determinations. All experiments were repeated at least three times to confirm the reproducibility of the observations.
The presence of a system of OPN-mediated regulation of NO in the islets and β-cells suggests potential targets for modulation of the NO-dependent components of the inflammatory response. The existence of OPN as a potential endogenous negative-feedback protective factor against STZ-induced NO in the islets is unique. A better understanding of OPN regulation and action during the early stages of diabetes and the factors involved in regulation of NO pathways will help to unravel the pathophysiology of diabetes and its associated complications.

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