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NO induces a cGMP-independent release of cytochrome *c* from mitochondria which precedes caspase 3 activation in insulin producing RINm5F cells

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Abstract Exposure of RINm5F cells to interleukin-1β and to several chemical NO donors such as sodium nitroprusside (SNP), SIN-1 and SNAP induce apoptotic events such as the release of cytochrome *c* from mitochondria, caspase 3 activation, Bcl-2 downregulation and DNA fragmentation. SNP exposure led to transient activation of soluble guanylate cyclase (sGC) and prolonged protein kinase G (PKG) activation but apoptotic events were not attenuated by inhibition of the sGC/PKG pathway. Prolonged activation of the cGMP pathway by exposing cells to the dibutyryl analogue of cGMP for 12 h induced both apoptosis and necrosis, a response that was abolished by the PKG inhibitor KT5823. These results suggest that NO-induced apoptosis in the pancreatic β-cell line is independent of acute activation of the cGMP pathway.

Key words: Nitric oxide; Pancreatic β-cell; Apoptosis; Cyclic GMP; Cytochrome *c*; Caspase 3

1. Introduction

Cytokines and free radicals produced by immune/inflammatory cells have been implicated as effector molecules of β-cell dysfunction and destruction in insulin-dependent diabetes mellitus (IDDM) [1–5]. Indeed, combinations of interleukin (IL)-1β, tumor necrosis factor-α and interferon-γ suppress islet function, damage DNA and decrease cell viability in rat and human pancreatic β-cells [6–10]. There is also evidence that apoptosis participates in the process of cytokine-induced β-cell death [11,12]. Most cytokine actions on β-cell damage involve the generation of nitric oxide (NO) (reviewed in [5]). NO inhibits mitochondrial respiration and induces Ca^{2+} efflux [13]. In addition NO modulates the mitochondrial permeability transition (PT), a process that is linked to release of apoptogenic factors such as cytochrome *c* [14]. It has recently been reported that the apoptotic actions of NO in insulin producing HIT-T15 cells are mediated by the sGC/PKG system [15]. In the present work, we have studied the role of mitochondria in NO-mediated apoptosis in rat insulinoma derived RINm5F cells and its regulation by the cGMP pathway.

2. Materials and methods

2.1. Materials

Cyclosporin A (CsA) was generously provided by Dr. S.F. Borel (Sandoz), IL-1β was from Genzyme, DEVD.AFC, Z.VAD.FMK, Z.YVAD.FMK, KT5823, protein kinase A inhibitor 6–22 amide and protein kinase G substrate-BPDEtide were from Calbiochem, cGMP (^{125}I) radioimmunoassay (RIA) system, PVDF membranes and [γ-^{32}P]ATP were from Amersham, cytochrome *c* antibody (7H8.2C12) was from Pharmingen, sodium nitroprusside (SNP) was from Merck, RPMI 1640 was from Biowhittaker, streptomycin, penicillin, glutamine, amphotericin B, cell death detection ELISA^{plus} and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc) were from Boehringer Mannheim, Bcl-2 antibody (DC21) was from Santa Cruz Biotechnology, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) was from Tocris Cookson, *S*-nitroso-*N*-acetylpenicillamine (SNAP) was from ICN, phosphocellulose paper (P-81) was from Whatman, N^6monomethyl-L-arginine (NMMA), γ-mouse and γ-rabbit peroxidase conjugate, trypsin, 3-morpholinosydnonimine (SIN-1) and other chemicals were from Sigma.

2.2. Cell culture

RINm5F cells were maintained in RPMI 1640 supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin G, 2.5 μg/ml amphotericin B, 2 mM glutamine, 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

2.3. Measurement of soluble guanylate cyclase (sGC) activity and cGMP-dependent protein kinase (PKG) activity

RINm5F cells (5×10^6) were homogenized in 50 μl of 20 mM HEPES pH 7.5, 10 mM EGTA, 40 mM glycerophosphate, 1% NP-40, 25 mM $MgCl_2$, 2 mM sodium orthovanadate, 140 mM NaCl, 1 mM DTT, and a mixture of protease inhibitors containing 1 mM Pefabloc, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Extracts were centrifuged for 15 min at $15\,000\times g$ and supernatants were used for enzyme measurements. sGC activity was measured as described in [16]. Briefly, aliquots of supernatants corresponding to 30 μg protein were incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 0.5 mM 1-methyl-3-isobutylxanthine, 7.5 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, and 1 mM GTP with or without 1 mM sodium nitroprusside. The reaction was terminated by addition of 0.05 N HCl (1:9) and boiling for 3 min. cGMP levels were measured by a commercial RIA according to the manufacturer's instructions. PKG activity was measured as in [17]. Aliquots of 10 μl of homogenate (containing 15 μg protein) were added to 50 μl of assay mixture containing 20 mM Tris-HCl pH 7.4, 200 μM ATP, 100 μM of protein kinase G substrate-BPDEtide, 20 mM $MgCl_2$, 100 μM 1-methyl-3-isobutylxanthine, 1 μM protein kinase A inhibitor 6–22 amide and 0.5 μCi [γ-^{32}P]ATP. The reaction was allowed to proceed at 30°C for 10 min. To terminate the reaction, 108 μl of 10% trichloroacetic acid was added and the mixture was then centrifuged for 2 min a $15\,000\times g$ to precipitate proteins, and supernatants were spotted onto phosphocellulose filters. After drying for 30 min, the filters were dropped into 75 mM phosphoric acid, washed with four changes of phosphoric acid to remove unreacted [γ-^{32}P]ATP, and once with ethanol. Filters were then dried and counted in a liquid scintillation counter to measure incorporation of ^{32}P into peptide.

2.4. Detection of histone-associated DNA

Cells were scraped off the plates and centrifuged at $700\times g$ for 10 min, washed with PBS and resuspended in lysis buffer. DNA fragmentation into nucleosomes was determined with the cell death detection ELISA^{plus} according to the manufacturer's instructions.

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2.5. Assay of cell toxicity

Cells were carefully collected from culture and centrifuged at 3000 rpm for 3 min. Supernatants were collected for analysis of lactate dehydrogenase (LDH) activity and pellets were lysed in 5 mM Tris (pH 7.0), 20 mM EDTA, 0.5% Triton X-100. LDH activity was assayed spectrophotometrically following the decrease in absorbance of NADH at 340 nm. The percentage of LDH released from cells to culture medium was calculated according to the following formula:

$$\%\ \text{LDH released} = \frac{\text{LDH in culture medium}}{\text{LDH in culture medium} + \text{LDH in cell lysates}} \times 100$$

2.6. Measurement of caspase activity

Cells were sedimented at 700×*g* for 3 min, washed twice in PBS, and resuspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA supplemented with a cocktail of protease inhibitors containing 1 mM Pefabloc, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. After 30 min incubation on ice, cells were lysed with 40 strokes in a homogenizer. Following removal of particulate matter by centrifugation at 15 000×*g* for 15 min, supernatants were supplemented with 0.5 mM EDTA and 2 mM DTT. 50 μg of cytosolic protein was added to 225 μl of assay buffer containing 25 mM HEPES (pH 7.5), 0.1% (w/v) CHAPS, 10 mM DTT, 100 U/ml aprotinin, 1 mM Pefabloc and 100 μM of the caspase substrate DEVD.AFC and incubated for 2 h at 37°C. The reaction was terminated by addition of 1.225 ml of ice-cold assay buffer. Fluorescence was measured using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Standards containing 0–1500 pmol of AFC were utilized to determine the amount of fluorochrome released.

2.7. Western blot analysis of cytochrome c and Bcl-2

Cells were collected by centrifugation at 700×*g* for 3 min at 4°C, washed twice with ice-cold PBS and centrifuged at 700×*g* for 3 min. For analysis of cytochrome *c*, cell pellets were resuspended in 60 μl of extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM HEPES pH 7.5, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 mM Pefabloc, 10 μg/ml pepstatin A and 10 μg/ml leupeptin. After incubation on ice for 30 min, cells were spun at 15 000×*g* for 15 min. Aliquots of 10 μg protein were resolved on 12.5% SDS-polyacrylamide gel and then blotted to PVDF membranes. Membranes were incubated for 6 h at room temperature with anti-cytochrome *c* monoclonal antibody 7H8.2C12 (diluted 1:2000). Following removal of the excess of primary antibody by three washes with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20, membranes were incubated with anti-mouse IgG conjugated with peroxidase (1:20 000) for 1 h. Bound antibodies were detected by enhanced chemiluminescence. For analysis of Bcl-2, cells were placed in 50 μl of buffer (20 mM Tris-HCl, 137 mM NaCl, 5 mM $MgCl_2$, 10% glycerol and 1% NP-40, 1 mM Pefabloc, 10 μg/ml pepstatin A and 10 μg/ml leupeptin). Lysis was carried out at 4°C for 30 min. Cell lysates were sonicated for 10 s on ice. Lysates were then centrifuged at 15 000×*g* for 15 min. 10 μg of cytosolic protein was resolved by SDS-PAGE electrophoresis as above and Bcl-2 was detected by immunoblotting and enhanced chemiluminescence.

3. Results

3.1. Effect of sodium nitroprusside on the GC/PKG system

Fig. 1 shows that RINm5F cells contain soluble guanylate cyclase activity that was stimulated by sodium nitroprusside. The magnitude of stimulation is dependent on previous exposure of cells to the NO donor. Thus, short periods of exposure of cells (0.5–1.0 h) to SNP slightly increased NO-activated enzyme activity; longer exposures (2.0–4.0 h) led to a substantial reduction in NO-activated enzyme activity. Basal enzyme activity was not significantly affected by exposure of cells to the NO donor. Table 1 shows that PKG is activated 10-fold after 0.5 h of exposure to 1 mM SNP. This activation remains stable after 12 h of exposure. The addition of cGMP to the assay system increased 10-fold PKG activity in extracts from control non-exposed cells, but failed to further enhance PKG activity in SNP-treated cells. The guanylate cyclase inhibitor ODQ fully blocked PKG activation induced by short (0.5 h) exposure to SNP, but failed to do this at longer periods of treatment (6.0–12.0 h).

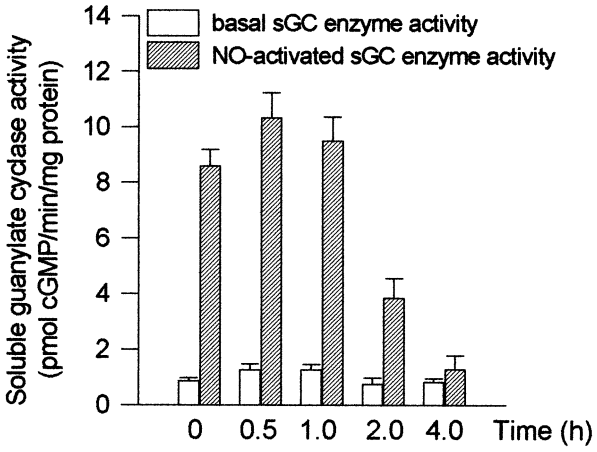

Fig. 1. Effect of SNP exposure on soluble GC activity in RINm5F cells. Homogenates from control untreated cells and from cells exposed to 1 mM SNP for 0.5, 1, 2, or 4 h were assayed for basal and SNP-activated enzyme activity as described in Section 2. Data are means ± S.D. from three independent experiments

3.2. Nitric oxide and cytochrome c release

Fig. 2 shows that the chemical NO donor SNP induces a time-dependent release of cytochrome *c* in RINm5F cells. This action was mimicked by other NO donors such as SNAP and SIN and by exposure of cells to IL-1β. The effect of IL-1β was fully reversed by the presence of the NO synthase inhibitor NMMA.

CsA, a specific inhibitor of the permeability transition pore (PT) opening in mitochondria, partially reversed the effect of SNP and IL-1β on cytochrome *c* release (Fig. 3).

3.3. Regulation of NO-dependent cytochrome c release

Fig. 4A shows that neither the inhibitor of guanylate cyclase ODQ nor the inhibitor of PKG KT5823 is able to counteract the actions of chemically generated NO on cytochrome *c* release from mitochondria. On the other hand, exposure of cells to the dibutyryl analogue of cGMP (dbcGMP) led to release of cytochrome *c* from mitochondria to cytosol. This

Table 1
Time course of NO-induced PKG activity in extracts from RINm5F cells

Time (h)	PKG activity (nmol/min/mg protein)		
	1 mM SNP		1 mM SNP+10 μM ODQ
	No addition	10 μM cGMP	
0	1.2 ± 0.19	11.6 ± 0.59[a]	1.2 ± 0.17
0.5	12.4 ± 0.42[a]	11.9 ± 1.15[a]	1.6 ± 0.83
6.0	9.2 ± 1.06[a,b]	9.7 ± 0.23[a,b]	9.5 ± 0.25[a,b]
12.0	10.5 ± 1.21[a,b]	10.4 ± 0.48[a,b]	9.4 ± 0.85[a,b]

Cells were exposed to either 1 mM SNP or 1 mM SNP and 10 μM ODQ for the indicated times and basal and cGMP-stimulated protein kinase G enzyme activity was measured as described in Section 2. When appropriate, ODQ was added 30 min before SNP. Data are means ± S.D. from three independent experiments. One-way ANOVA was used for multiple group comparisons.
[a]Significantly greater than 0 h ($P < 0.05$).
[b]Significantly greater than 0.5 h ($P < 0.05$).

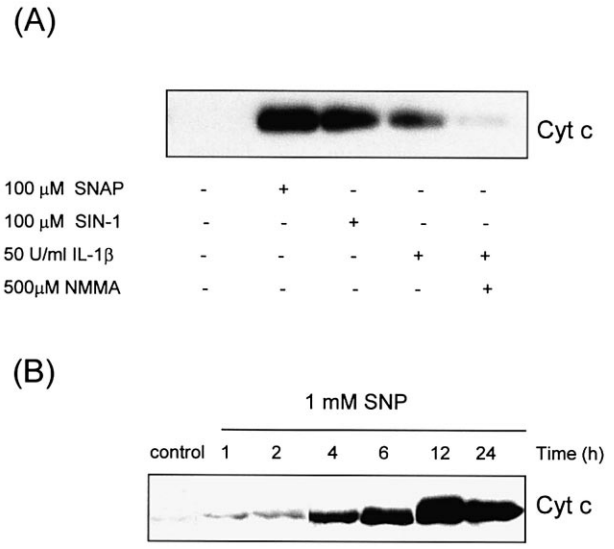

Fig. 2. NO-induced release of cytochrome *c* from mitochondria. A: Release of cytochrome *c* induced by exogenous NO and by IL-1β. Cells were exposed to SNAP and SIN-1 for 15 h and to IL-1β and to IL-1β+NMMA for 24 h. B: Time course of SNP-induced release of cytochrome *c*. Cytosolic extracts were prepared at the indicated times following exposure to SNP. Immunoblots are representative of three independent experiments.

action was fully prevented by KT5823. Similar results were obtained when NO was endogenously generated following exposure of cells to IL-1β (Fig. 4B). In addition, both SNP- and IL-1β-induced cytochrome *c* release were partially prevented by the caspase inhibitor Z.VAD.FMK (Fig. 4C). The relationship between caspases and cytochrome *c* release from mitochondria is shown in Fig. 5A. Exposure of cells to 1 mM SNP led to caspase 3 activation in a time-dependent manner and inhibition of mitochondrial PT with CsA partially prevented this effect. Fig. 5B shows that endogenous generation of NO following exposure of cells to IL-1β led to a 10-fold increase in caspase 3 activity that was not dependent on the cGMP system.

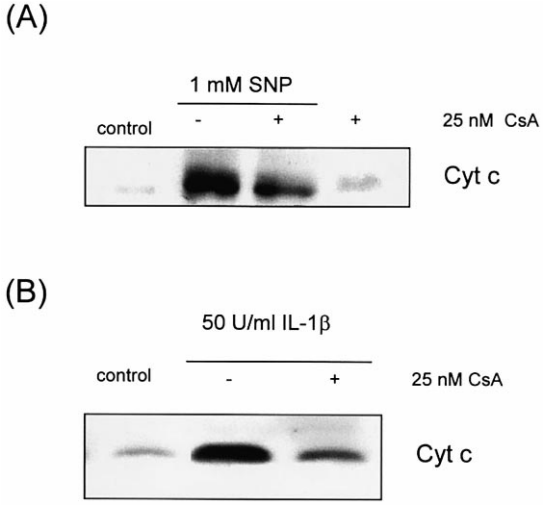

Fig. 3. Inhibition of SNP- and IL-1β-induced cytochrome *c* release by CsA. When appropriate, CsA was added 1 h before addition of SNP and IL-1β and cells were cultured for 12 h (A) and 18 h (B). Immunoblots are representative of three independent experiments.

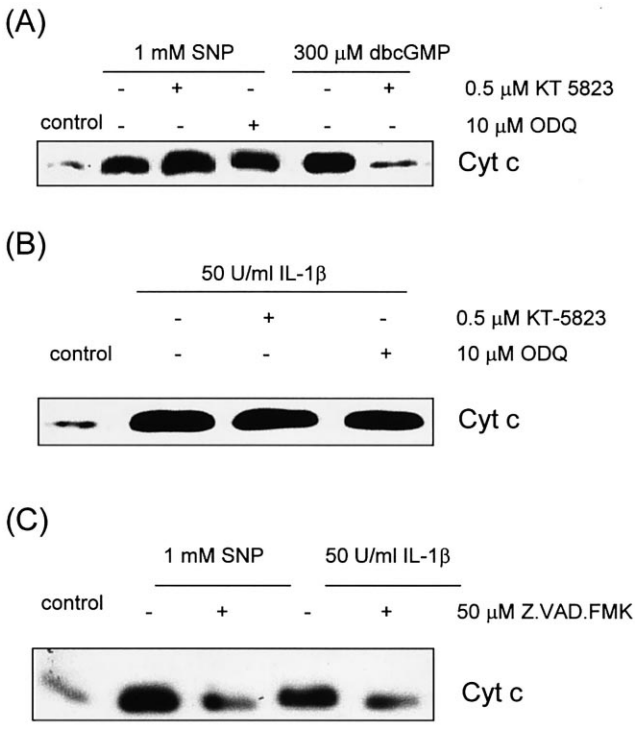

Fig. 4. Role of cGMP and caspases on NO-induced release of cytochrome *c*. A: SNP- and cGMP-induced release of cytochrome *c*. ODQ and KT5823 were added 30 min before addition of SNP and dbcGMP. Cells were then cultured for 12 h. B: IL-1β-induced release of cytochrome *c*. ODQ was added 30 min before addition of IL-1β. Cells were then cultured for 24 h. C: Effect of Z.VAD.FMK on NO-induced cytochrome *c* release from mitochondria. Z.VAD.FMK was added 30 min before the addition of 1 mM SNP and IL-1β. Cells were then cultured for 12 and 18 h respectively. Immunoblots are representative of three independent experiments.

3.4. NO and DNA damage and cell necrosis

Fig. 6 shows that both SNP- and IL-1β-induced DNA fragmentation were not inhibited by either ODQ or KT5823. By contrast, dbcGMP-induced DNA fragmentation was fully prevented by KT5823. SNP, IL-1β and dbcGMP stimulated the release of LDH to medium. Inhibition of the sGC/cGMP pathway with ODQ and KT5823 blocked this action.

3.5. NO and Bcl-2 degradation

The effect of SNP and dbcGMP on Bcl-2 levels is shown in Fig. 7. SNP exposure led to a time-dependent downregulation of the protein. The action of SNP was fully prevented by addition of the caspase inhibitor Z.VAD.FMK. By contrast, the protein kinase G inhibitor KT5823 failed to block the effect of SNP. Exposure of cells to dbcGMP for 12 h led to a decrease in Bcl-2 protein levels that was fully counteracted by KT5823.

4. Discussion

Evidence has been accumulated over the past years concerning the role of apoptosis in the control of β-cell population and in the pathogenesis of IDDM [11,12,18–21]. Apoptotic rodent β-cell death seems to be mediated by the generation of NO in a process regulated by inflammatory cytokines [10,18–22]. The results of this study indicate that NO activates caspase 3 in rat insulinoma-derived RINm5F

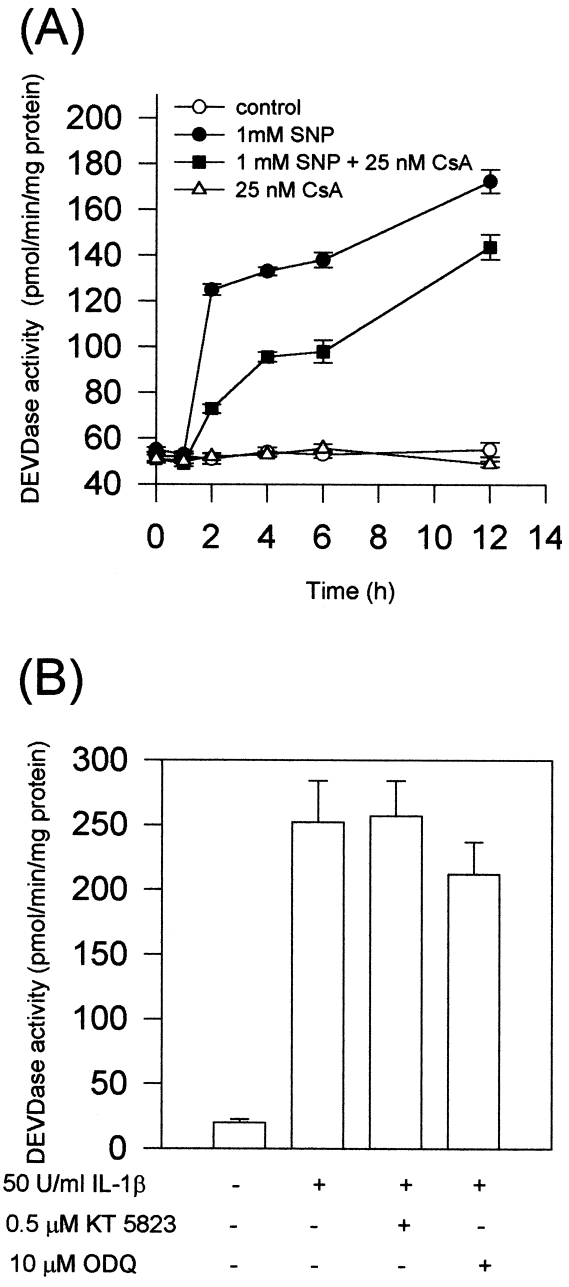

Fig. 5. Effect of PT and cGMP pathway inhibitors on NO-dependent caspase 3 activation. A: Effect of CsA on NO-induced caspase 3 activation. When appropriate, CsA was added 1 h before time 0. B: Effect of cGMP pathway inhibitors on IL-1β-induced caspase 3 activation. Cells were exposed to IL-1β for 24 h in the presence of ODQ and KT5823 respectively. Data are means ± S.D. from 3–4 independent experiments.

cells and that this event is preceded by release of cytochrome *c* from mitochondria in a cGMP-independent manner. The study also shows that NO-induced DNA fragmentation is cGMP-independent.

The actions of NO on apoptosis depend on cell type, concentration, interaction with other radicals, and the redox state of cells [23–30]. In cell systems where NO is anti-apoptotic, the cGMP pathway is involved [31–35]. Pancreatic β-cell apoptosis is controlled by cGMP in a different manner. It has been reported that cGMP is involved in NO-induced apoptosis [15]. In fact, our data indicate that RINm5F cells are endowed with a GC/PKG system and that exposure of cells to the NO donor SNP leads to initial activation of GC followed by a decay with longer periods of treatment. These results are in agreement with those reported in smooth muscle cells, where a decrease in GC mRNA stability induced by NO has been described [36,37]. On the other hand, NO exposure led to a sustained increase in PKG activity that was resistant to blockade of GC with ODQ. It is entirely possible that chronic exposure to NO leads to PKG activation by cGMP-independent mechanisms such as phosphorylation [38,39]. Our results also indicate that NO exposure leads to necrotic cell death and apoptosis and that these actions were mimicked by dbcGMP. PKG inhibitors counteracted the actions of

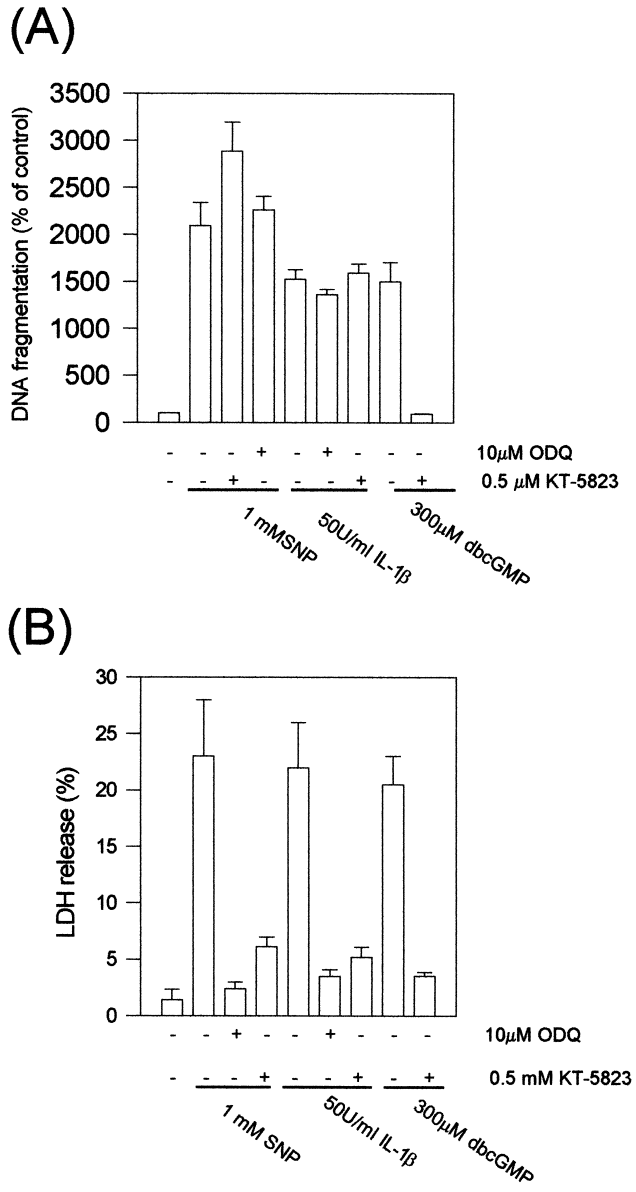

Fig. 6. NO-induced apoptosis occurs in a cGMP-independent manner. ODQ and KT5823 was added 30 min before addition of SNP, IL-1β and dbcGMP. Cells were then cultured for 12 h (SNP- and dbcGMP-treated cells) and for 24 h (IL-1β-treated cells). A: DNA fragmentation into nucleosomes. B: Percentage of LDH release to medium. Data are means ± S.D. from 3–4 independent experiments.

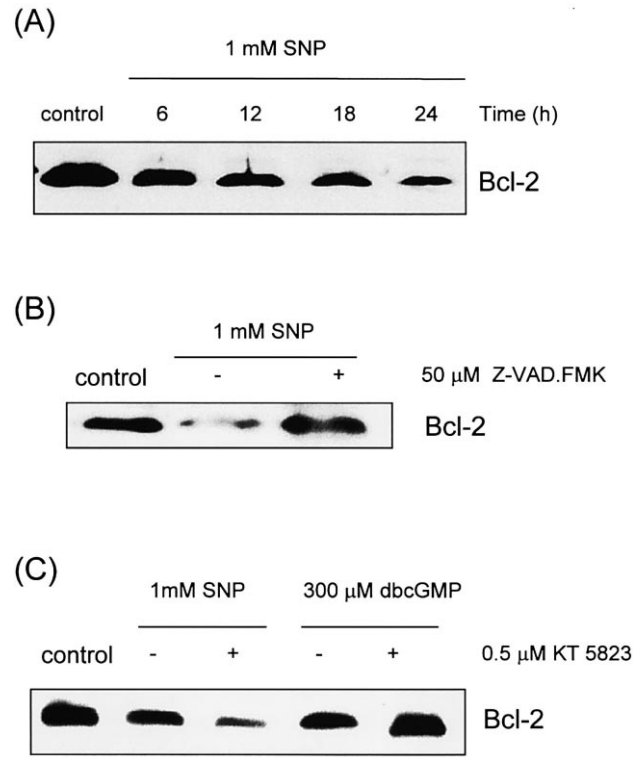

Fig. 7. Effect of NO and dbcGMP on Bcl-2 protein levels. A: Time dependence of Bcl-2 degradation induced by 1 mM SNP. B: Effect of Z.VAD.FMK on Bcl-2 degradation induced by SNP. Cells were cultured for 24 h with the additions given in the figure. C: Effect of KT5823 on SNP- and dbcGMP-induced degradation. Cells were cultured for 12 h with the additions given in the figure. Immunoblots are representative of three independent experiments.

dbcGMP on apoptotic cell death and necrosis, but they failed to block the actions of NO on apoptosis. These findings speak in favor of the notion that NO triggers apoptosis in RINm5F cells in a cGMP-independent manner. We also found that NO-induced LDH release is cGMP-dependent. This is in agreement with cell viability studies performed in HIT-T15 cells and suggests that cGMP might control processes that lead to necrosis in pancreatic cells [15].

Cytochrome *c* release from mitochondria has been involved in the control of caspase activation during apoptosis [40–46]. NO generated both by chemical donors and by exposure of cells to IL-1β provoked the release of cytochrome *c* from mitochondria in RINm5F cells. In fact, this organelle seems to play an important role in controlling NO-induced apoptosis in RINm5f cells since NO-induced caspase 3-like activation and DNA fragmentation were preceded by the appearance of cytochrome *c* in cytosol. It is now known that the activation of effector caspases and nuclear apoptosis is controlled by apoptogenic factors such as cytochrome *c* released from mitochondria following opening of PT pores [14,46–50]. The finding that the inhibitor of the PT pore CsA partially counteracted NO-induced apoptotic events in these cells supports the notion that NO triggers mitochondrial PT [51].

The possibility that NO-induced mitochondrial PT was secondary to Bcl-2 degradation was tested with the protease inhibitor Z.VAD.FMK. Whereas Z.VAD.FMK fails to fully counteract the release of cytochrome *c*, it prevents Bcl-2 downregulation. This suggest that Z.VAD.FMK-inhibited proteases participate in the exacerbation of the actions of NO at the mitochondrial level.

It has been shown that low concentrations of NO increase the expression of Bcl-2 protein in a cGMP-dependent manner and prevent apoptosis in some cell systems [51]. Although a similar action in RINm5F cells cannot be ruled out, our results indicated that the apoptotic action of NO at high concentrations depends on Bcl-2 downregulation and is secondary to caspase activation. Besides, the finding that inhibition of PKG with KT5823 counteracted cGMP-induced Bcl-2 downregulation but failed to block NO-induced Bcl-2 downregulation indicates that the cGMP/PKG system is not involved in the action of NO on Bcl-2 levels. According to its pleiotropic nature, NO can trigger alternative apoptotic pathways such as the upregulation of p53 and CD95/Fas receptor proteins [24,26,34,52,53].

Our data suggest that mitochondrial handling of key apoptotic events in insulin producing cells is affected by NO in a cGMP-independent manner. Future studies will be required to elucidate the signaling pathways that mediate the action of NO in mitochondria.

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