Evidence for the involvement of cGMP and protein kinase G in nitric oxide-induced apoptosis in the pancreatic B-cell line, HIT-T15

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Received 8 October 1996; revised version received 21 November 1996

Abstract Intracellular production of nitric oxide (NO) is thought to mediate the pancreatic B-cell-directed cytotoxicity of cytokines in insulin-dependent diabetes mellitus, and recent evidence has indicated that this may involve induction of apoptosis. A primary effect of NO is to activate soluble guanylyl cyclase leading to increased cGMP levels and this effect has been demonstrated in pancreatic B-cells, although no intracellular function has been defined for islet cGMP. Here we demonstrate that the NO donor, GSNO, induces apoptosis in the pancreatic B-cell line HIT-T15 in a dose- and time-dependent manner. This response was significantly attenuated by micromolar concentrations of a specific inhibitor of soluble guanylyl cyclase, ODQ, and both 8-bromo cGMP (100 μM) and dibutyryl cGMP (300 μM) were able to fully relieve this inhibition. In addition, incubation of HIT-T15 cells with each cGMP analogue directly promoted cell death in the absence of ODQ. KT5823, a potent and highly selective inhibitor of cGMP-dependent protein kinase (PKG), abolished the induction of cell death in HIT cells in response to either GSNO or cGMP analogues. This effect was dose-dependent over the concentration range of 10–250 nM. Overall, these data provide evidence that the activation of apoptosis in HIT-T15 cells by NO donors is secondary to a rise in cGMP and suggest that the pathway controlling cell death involves activation of PKG.

Key words: Pancreatic B-cell; Guanidine cyclase; Protein kinase G; Apoptosis; Nitric oxide

1. Introduction

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterised by macrophage and T-cell infiltration of the endocrine pancreas, resulting in insulitis and specific destruction of islet B-cells. The cytotoxic attack is mediated by cytokines (especially interleukin 1-β) secreted by the infiltrating immune cells. There is a considerable body of evidence suggesting that cytokine-induced death of pancreatic B-cells derives from the induction of nitric oxide synthase (iNOS) leading to generation of NO within the cells (reviewed in [1]; see also [2–5]). NO then interacts with a variety of intracellular targets which contain either haem groups or iron-sulphur centres, including respiratory carries and metabolic enzymes [6]. The functional consequences of these interactions may then mediate some of the cytotoxic effects of the molecule.

In a number of cell types, including macrophages, thymocytes and chondrocytes [7–9], recent evidence has suggested that generation of NO can lead to induction of cell death by activation of apoptosis. Moreover, it is now evident that cytokines and a variety of chemical NO donors also induce apoptosis in freshly isolated rat islets and in clonal pancreatic B-cell lines [10–13][1]. The mechanisms by which this occurs remain undefined, although NO is known to cause direct damage to DNA by deamination of purines and pyrimidines [14], in addition to exerting inhibitory effects on metabolic enzymes. A further well-characterised effect of NO is the activation of soluble guanylyl cyclase (GC), leading to increased levels of intracellular cGMP [15–17] and subsequent activation of a cGMP-dependent protein kinase, PKG. Pancreatic islets express GC [18] but the role of cGMP in islet cell signalling remains enigmatic [19,20]. Indeed, the cumulative evidence indicates that modulation of cGMP levels is not involved in the acute regulation of insulin synthesis or secretion [21].

In the present work, we have investigated the possibility that a hitherto unrecognised function of the GC-PKG system in pancreatic B-cells may be to regulate the entry of these cells into the apoptotic pathway. The glucose-responsive B-cell line, HIT-T15, was induced to enter apoptosis by incubation with the NO donor, GSNO and the results revealed that GSNO-induced apoptosis was significantly attenuated by a specific inhibitor of GC, ODQ. Cell permeant cGMP analogues were able to fully restore GSNO-induced apoptosis in the presence of ODQ. Furthermore, analogues of cGMP were also able to induce cell death directly in HIT-T15 cells, a response which was abolished by the selective PKG inhibitor KT5823. This agent also dose-dependently inhibited the induction of apoptosis by GSNO. This suggests that, among the numerous reported effects of NO, activation of the cGMP pathway is of paramount importance in the induction of apoptosis in pancreatic B-cells.

2. Experimental procedures

HIT-T15 cells were seeded at 1×10^5 cells/well in 24-well plates in RPMI-1640 medium supplemented with l-glutamine (300 μg/ml), sodium penicillin G (100 IU/ml), streptomycin sulphate (100 μg/ml) and foetal calf serum (10%) and incubated in a air/CO_2 atmosphere (95:5%), with 100% humidity, for 48 h. Cells were then treated with test reagents for 1–24 h, after which time detached cells were harvested by centrifugation of the medium (500×*g*/2 min). In experiments involving co-incubation with apoptosis-inducing agents (GSNO or cGMP analogues) and the enzyme inhibitors ODQ or

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Abbreviations: GC, guanylyl cyclase; GSNO, S-nitrosoglutathione; iNOS, inducible nitric oxide synthase; NO, nitric oxide; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PKG, cGMP-dependent protein kinase

[1] M. Dimatteo, A.C. Loweth, S. Thomas, N.G. Morgan, J. Thorpe and I.C. Green. Manuscript submitted.

PII S0014-5793(96)01392-0

KT5823, cells were pre-incubated with the inhibitors for 30 and 60 min, respectively. Dead cells counts were carried out using vital dye (Trypan blue) exclusion and heamocytometry. Apoptosis was confirmed by electron microscopy, DNA gel electrophoresis and fluoromicroscopic examination of acridine orange-stained cells, using the methods described by Morgan et al. [22]. Statistical analysis was carried out using 1-way analysis of variance and Tukey's test of least significant difference.

3. Results and discussion

In agreement with previous work using other NO donors [10,11], GSNO was found to induce apoptosis in the B-cell line, HIT-T15. The concentrations of GSNO required to induce HIT cell death were low compared to those employed in other cell types, with as little as 100 μM GSNO promoting a significant increase in cell death compared to untreated controls (Fig. 1). Many chemical NO donors are thought to have a relatively short half-life after hydration, but GSNO differs in that it induces a sustained, low-level production of NO for several hours in tissue culture medium (R.D. Hurst, personal communication). The significance of this for the time course of induction of apoptosis is unclear but increased cell death only became evident following exposure of HIT cells to GSNO for 3–5 h (Fig. 2). Apoptosis is known to require de novo protein synthesis in many cell types and the time-course observed for the onset of death in these experiments is consistent with this mechanism in pancreatic B-cells. Examination of the cells by acridine orange staining and fluoromicroscopy revealed that at least 75–80% of the detached cells from each condition showed evidence of chromatin condensation and margination, indicating that the cells had died by apoptosis (not shown). Taking this with the 5–20-fold increase in detached, dead cell numbers resulting from GSNO treatment, quantified by vital staining, it can be seen that GSNO consistently induced a significant increase in the number of apoptotic cells, compared to untreated controls. Cells that remained attached to the substrate were found to be viable, suggesting that detachment is an early event in the onset of

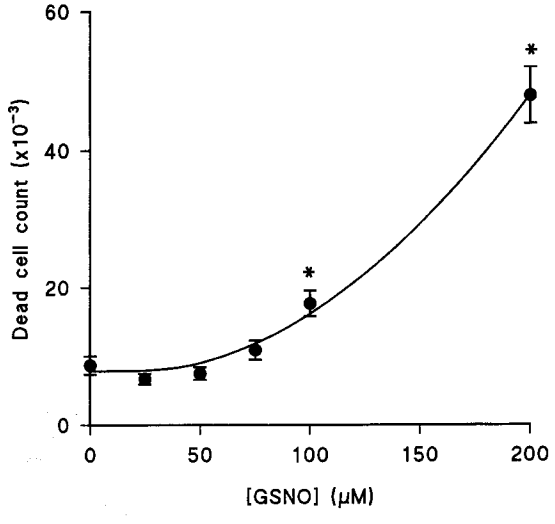

Fig. 1. Dose-response relationship for the induction of cell death by GSNO. HIT-T15 cells were cultured for an initial period of 48 h and then exposed to increasing concentrations of GSNO (0–200 μM). Detached cells were harvested and the numbers of non-viable cells determined by vital staining. Each point represents the mean dead cell count ± SEM ($n = 8$). *$P < 0.001$ compared to control.

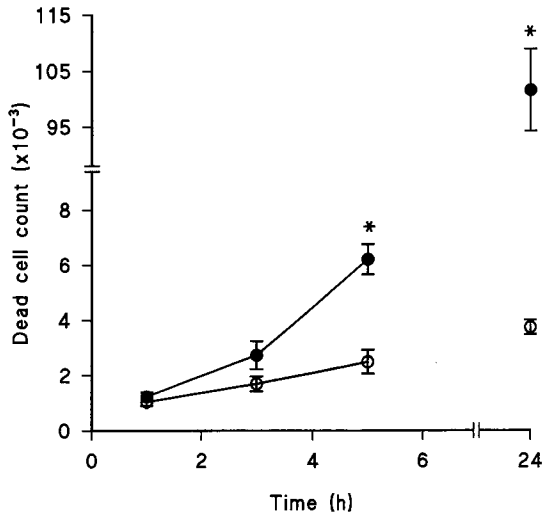

Fig. 2. Time course of cell death induced by GSNO in HIT-T15 cells. HIT-T15 cells were cultured for 48 h and then exposed to 200 μM GSNO for a further 1–24 h. Detached cells were harvested and the numbers of non-viable cells determined by vital staining. Each point represents the mean of 6 replicates ± SEM. *$P < 0.001$ compared to control.

apoptosis in B-cells. This observation is consistent with the recognised progression of apoptosis, where retraction of extracellular contacts is an early event [23]. The mechanism of cell death was confirmed as apoptosis by two further methods. Electron microscopy revealed that cells exposed to GSNO exhibited morphological features of apoptosis (Fig. 3A) and DNA was extracted from GSNO-treated cells prior to examination by agarose-gel electrophoresis. The DNA exhibited an oligonucleosomal laddering pattern that is typical of cells dying by apoptosis (Fig. 3B).

Since NO donors are well known to promote a rise in cGMP levels by virtue of their ability to cause activation of soluble GC, HIT cells were treated with cell permeant analogues of cGMP (8-bromo-cGMP; dibutyryl cGMP) and their responses monitored. Both analogues caused a marked increase in HIT cell death during 24 h exposure (100 μM 8b-cGMP, 296 ± 20%; 300 μM dbcGMP, 228 ± 15% vs. control; $P < 0.001$; see also Fig. 6) suggesting that elevation of cGMP may be a primary component of the signalling pathway leading to activation of apoptosis in pancreatic B-cells. In order to examine this possibility further, cells were exposed to GSNO in the presence of a specific inhibitor of soluble GC, ODQ [24]. This agent failed to alter the extent of cell death in control cells but significantly inhibited the capacity of 200 μM GSNO to cause HIT cell death. Over several experiments, 10 μM ODQ exerted a consistent 60–65% inhibition of the response to GSNO. Addition of either 8-bromo-cGMP or dibutyryl cGMP to cells incubated with GSNO totally prevented the inhibitory effect of ODQ, confirming the involvement of cGMP in the death response (Fig. 4).

The actions of cGMP can be mediated by several intracellular target proteins including certain ion channels, phosphodiesterase enzymes and cGMP-dependent protein kinases. In order to examine which of these is involved in the induction of apoptosis by cGMP in pancreatic B-cells, HIT cells were treated with a highly selective inhibitor of PKG, KT5823 [25–27]. This agent did not influence the viability of control

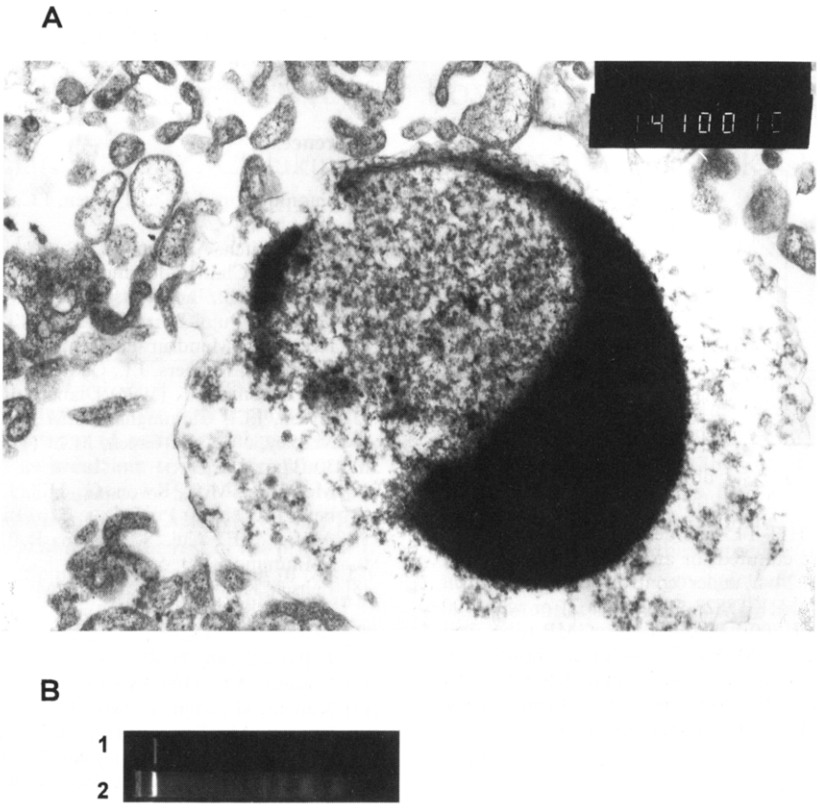

Fig. 3. Evidence for induction of apoptosis in GSNO-treated HIT-T15 cells. A: Following 24 h exposure to GSNO, detached HIT-T15 cells were harvested and fixed with gluteraldehyde prior to processing for electronmicroscopy. A typical example is shown in which marked condensation and margination of chromatin is evident. These features are indicative of death by apoptosis. B: HIT-T15 cells were cultured for 24 h in the presence (lane 2) or absence (lane 1) of 200 μM GSNO. Approximately 10^6 detached cells were harvested and lysed prior to DNA extraction. The DNA was electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualised under UV light.

cells but it potently inhibited the induction of cell death by GSNO. A significant effect was observed with as little as 50 nM of the inhibitor and complete abolition of the response to GSNO was seen with 250 nM KT5823 (Fig. 5). Strikingly, KT5823 also totally prevented the induction of HIT cell death by cGMP analogues (Fig. 6) indicating that both GSNO and

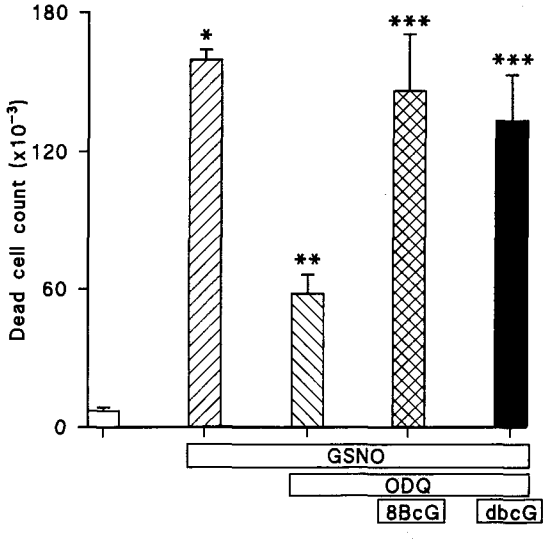

Fig. 4. Inhibition of GSNO-induced cell death by ODQ and relief by cGMP analogues. HIT-T15 cells were incubated for 24 h in the absence (open bar) or presence (shaded bars) of 200 μM GSNO. 10 μM ODQ was introduced (bars 3–5) and further supplemented with either 100 μM 8-bromo-cGMP (lane 4) or 300 μM dibutyryl cGMP (lane 5). Detached cells were harvested and the number of dead cells quantified by vital staining. Each point represents the mean of 6 replicates ±SEM. *$P < 0.001$ compared to control. **$P < 0.001$ relative to GSNO alone. ***$P < 0.001$ relative to GSNO+ODQ.

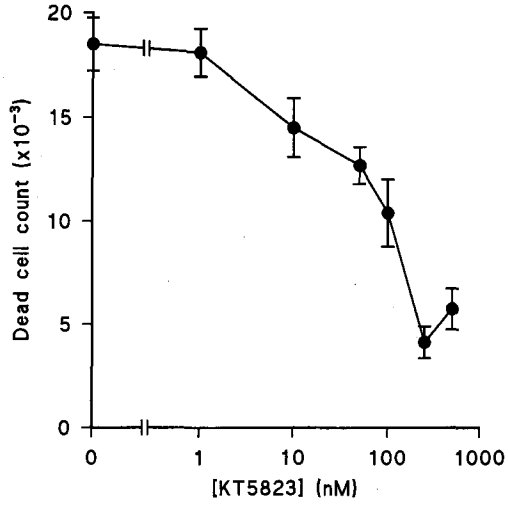

Fig. 5. Inhibition of GSNO-induced cell death by the protein kinase G inhibitor, KT5823. HIT-T15 cells were cultured for 48 h, then pre-incubated for 1 h with varying concentrations of KT5823 (0–500 nM). Cells were then exposed to 200 μM GSNO for a further 5 h. Detached cells were harvested and the number of non-viable cells quantified by vital staining. All concentrations of KT5823 between 50–250 nM significantly inhibted GSNO-induced cell death ($P < 0.001$ compared to GSNO alone; $n = 4$–8).

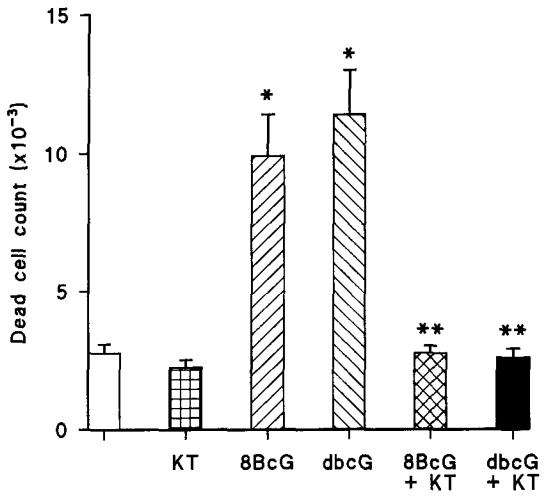

Fig. 6. Effect of KT5823 on HIT-T15 cell death induced by cGMP analogues. HIT-T15 cells were cultured for an initial period of 48 h, then incubated for a further 24 h under control conditions (open bar), in the presence of 500 nM KT5823 alone (bar 2) or with 100 μM 8-bromo-cGMP (8bcG) or 300 μM dibutyryl cGMP (dbcG) in the absence or presence of 500 nM KT5823 (KT) as shown. Detached cells were harvested and the dead cell count determined by vital staining ($n=6$). *$P<0.001$ compared to untreated cells. **$P<0.001$ compared to each cGMP analogue alone.

the cGMP analogues require activation of PKG in order to induce HIT cell death.

Overall, the results of the present study provide evidence that activation of apoptosis in pancreatic B-cells can occur in response to agents which promote a rise in cGMP levels. The data are consistent with the novel possibility that generation of cGMP and subsequent activation of PKG represent important components in a signalling pathway controlling apoptosis in B-cells. The downstream effectors by which this pathway induces cell death remain to be identified but it may be significant that PKG has recently been identified as an important regulator of the transcription of certain early response genes such as *c-fos* which are often expressed in cells entering apoptosis and are involved in control of cell growth and differentiation [26,28]. In this context, it is noteworthy that treatment of islet cells with cytokines (e.g. interleukin 1-β) leads to a rise in cGMP [29] and an increase in c-fos expression [30,31]. The temporal relationship between these events requires further study but the possibility should not be excluded that cGMP may be central to the cytotoxic actions of cytokines in islet cells. In a still wider context, the recent findings that cGMP-dependent pathways are involved in regulation of cell senescence and growth arrest in a number of cell types [26,32] suggest that the activation state of PKG may be an important regulatory factor which determines the ultimate fate of a cell according to the prevailing cGMP level. Furthermore, if, as suggested by the present results, the cGMP-PKG pathway proves to be crucially involved in the regulation of islet cell death, then manipulation of this pathway may offer a unique therapeutic route to intervene in the early loss of B-cells associated with progression towards insulin-dependent diabetes mellitus.

Acknowledgements: We are grateful to Scotia Pharmaceuticals and Lilly Industries for financial support of this work. Thanks are also due to Dr. Irene Green and Dr. Roger Hurst for their helpful comments and discussion.

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