Effects of the Imidazoline Binding Site Ligands, Idazoxan and Efaroxan, on the Viability of Insulin-Secreting BRIN-BD11 Cells

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ABSTRACT

Context Certain imidazoline drugs stimulate insulin secretion acutely but their longer term effects on the viability of pancreatic beta-cells are less well characterised. Indeed, some reports have suggested that imidazolines can be toxic to beta-cells while others have reported protective effects against other cytotoxic agents.

Objective In order to address these discrepancies, the effects of two structurally related imidazolines, efaroxan and idazoxan, on the viability of clonal BRIN-BD11 beta-cells, were compared.

Design and main outcome measures BRIN-BD11 cells were exposed to test reagents and their viability monitored by measuring cellular reducing ability and DNA fragmentation. Nitric oxide was measured indirectly via medium nitrite formation.

Results Efaroxan (up to 100 µM) did not directly affect BRIN-BD11 cell viability in the absence of other agents and it did not protect these cells against the cytotoxic effects of interleukin-1beta. Indeed, analysis of DNA fragmentation in BRIN-BD11 cells revealed that efaroxan enhanced the level of damage caused by interleukin-1beta. Idazoxan caused a time- and dose-dependent loss of BRIN-BD11 cell viability in the absence of other ligands. This was associated with marked DNA degradation but was not associated with formation of nitric oxide. The effects of idazoxan were insensitive to blockade of alpha2-adrenoceptors or 5-HT₁A (5-hydroxytryptamine; serotonin) receptors.

Conclusions The results confirm that idazoxan is cytotoxic to beta-cells but show that efaroxan is better tolerated. However, since efaroxan enhanced the cytotoxic effects of interleukin-1beta, it appears that this imidazoline may sensitize BRIN-BD11 cells to the damaging effects of certain cytokines.

INTRODUCTION

It is now well accepted that a range of imidazoline drugs (including compounds such as efaroxan, RX871024, phentolamine, antazoline) can stimulate insulin secretion and that members of this class may be useful as orally active compounds suitable for the management of type 2 diabetes [reviewed in 1, 2, 3, 4, 5]. Despite this, the mechanisms involved in their stimulatory effects have not been defined fully and increasing evidence indicates that the precise mechanism(s) may even be variable for each compound. Nevertheless, a consensus has emerged that two principal actions are likely to play a role. Firstly, the majority of imidazolines that stimulate insulin secretion can block ATP-sensitive potassium channels leading to membrane depolarisation and Ca influx [6, 7, 8, 9, 10, 11]. Secondly, some members of the class cause direct activation of the more distal
events involved in control of insulin exocytosis and, at least for some compounds, this latter effect may be of greater importance for their overall secretory activity [12, 13, 14]. It follows from this that members of the latter group probably interact with a critical intracellular binding site involved in the control of insulin secretion. This site is likely to be a member of the wider class of imidazoline binding sites defined in other tissues [2, 3, 15] but the molecule present in the beta-cell exhibits an atypical pharmacology [1, 5, 16]. It remains an important objective to define this site and it is encouraging that candidate molecules are now beginning to emerge [17].

In addition to these aspects, recent studies have added a new dimension to the potential utility of imidazoline compounds by providing evidence that some of them can also alter cell viability [18, 19, 20, 21]. Most strikingly, it has been reported that the loss of viability resulting from exposure of ob/ob mouse [19] or normal rat [21] pancreatic islets to the cytokine interleukin-1beta (IL-1beta) can be minimised by culture in the presence of certain imidazolines. Since IL-1beta is implicated as a causative agent in the loss of beta-cells seen in type 1 diabetes [22], this has raised the exciting possibility that imidazolines may also be therapeutically effective in preventing this condition. These observations also suggest the intriguing possibility that an imidazoline binding site may regulate the sensitivity of beta-cells to cytotoxic stimuli as well as controlling insulin secretion.

In considering the implications of these data, it is already evident, however, that the influence of imidazoline compounds on beta-cell viability varies dramatically, and that some (including idazoxan, phentolamine and antazoline) cause the death of proliferating beta-cells rather than exerting any protective influence [18]. In addition, morphological evidence indicates that idazoxan can also damage the fully differentiated beta-cells present in isolated islets [20]. Thus, there is still considerable uncertainty about the effects of imidazolines on beta-cell viability and, in the present study, we have evaluated further the influence of two structurally-related imidazolines, efaroxan and idazoxxan, on this parameter. We have also investigated whether efaroxan can alter the cytotoxic effects of IL-1beta in clonal BRIN-BD11 beta-cells.

**MATERIALS AND METHODS**

Clonal BRIN-BD11 cells ([23] passages 25-35) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, penicillin G (100 IU/mL) and streptomycin sulphate (100 µg/mL). Medium nitrite accumulation was measured as an index of NO formation by the cells. For these measurements, BRIN-BD11 cells were seeded into 96 well tissue culture plates and treated with test reagents for 24-48 h, as appropriate. After incubation, samples of the medium were harvested for measurement of nitrite formation. They were incubated with a mixture (1:1 vol:vol) of 1% sulphanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid and the optical density determined at 540 nm after colour development. Nitrite levels were determined by reference to a standard curve constructed using sodium nitrite.

The viability of BRIN-BD11 cells was determined by measuring the ability of the cells to reduce the dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) in the presence of the electron coupling reagent, phenozine methosulphate (PMS). Cells were grown in 96 well plates and, following incubation with test reagents, were exposed to a combination of MTS and PMS (formulated in the CellTiter 96® AQueous Assay reagent (Promega, Southampton, UK)) according to the manufacturer's instructions. Following a further incubation period (2-3 h at 37 °C) the extent of MTS reduction was determined by measuring the absorbance at 490 nm.

To monitor the integrity of cellular DNA, cell cycle analysis was performed. BRIN-BD11 cells were harvested by brief centrifugation (3 min; 1000 g) after treatment with test reagents.
and incubation with trypsin/EDTA. The medium was removed and the cells were washed with phosphate buffered saline (PBS), re-centrifuged and then fixed by resuspension in 2 mL of a mixture of ice cold ethanol: PBS (7:3 vol:vol). DNA integrity was determined by fluorescence activated cell counting after labelling with propidium iodide. Cell cycle analysis was carried out under contract by Babraham Technix (Babraham Bioscience Technologies Limited, Cambridge, UK).

Efaroxan was purchased from Tocris (Bristol, UK), idazoxan and S-nitrosoglutathione from Sigma (Poole, Dorset, UK). Interleukin-1beta was from Calbiochem (Nottingham, UK).

STATISTICAL ANALYSIS

Statistical analysis of results was performed by analysis of variance and differences were considered significant when two-tailed P was less than 0.05.

RESULTS

Previous studies have indicated that imidazoline drugs having closely related structures can exert profoundly differing effects on the viability of pancreatic beta-cells [18, 19, 20, 21]. These observations have been confirmed in the present work which revealed that exposure of the beta-cell line, BRIN-BD11, to idazoxan for 24 h was associated with a dose-dependent loss of MTS reduction (Figure 1). By contrast, exposure to efaroxan (up to 100 µM) was much less effective at attenuating MTS reduction by BRIN-BD11 cells, although modest inhibition was occasionally seen (e.g. Figure 2). Despite this, neither cell proliferation, insulin secretion rate nor the overall extent of cell viability (as judged by vital dye staining) was compromised by exposure to efaroxan for up to 7 days (not shown). In order to differentiate between possible effects of idazoxan on cell proliferation and on cell death (either of which could result in lowered MTS reduction after incubation) BRIN-BD11 cells were exposed to idazoxan (100 µM) for 24 h and then analysed by fluorescence activated cell sorting (FACS®; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to monitor the integrity of the cellular DNA (Figure 3). The DNA of control cells was principally distributed within a single G1 peak (Figure 3) although, as expected, there was also evidence that some cells were undergoing mitosis since a small G2 peak (representing fully replicated DNA) was seen. By contrast, after treatment with idazoxan, BRIN-BD11 cells displayed a markedly reduced G1 peak accompanied by significant DNA fragmentation as evidenced by the appearance of a large pre-G1 peak (Figure 3). This suggests that idazoxan directly promotes
the death of BRIN cells. Cells treated with efaroxan alone displayed a normal distribution of cellular DNA (see Figure 4).

To investigate the time course of these effects, BRIN cells were exposed to 100 μM idazoxan and harvested after 12, 16 and 20 h. The fragmentation of cellular DNA was then estimated by measuring the accumulation of DNA within the pre-G1 peak (Figure 5). Idazoxan caused a significant increase in DNA fragmentation as early as 12 h after exposure (P=0.006) and the extent of this increased significantly (P=0.010) in a time-dependent manner such that, after 20 h, almost all of the cellular DNA was fragmented. These results indicate that idazoxan is acutely toxic to beta-cells but confirm that its close structural analogue, efaroxan, is better-tolerated. One difference in pharmacological profile between idazoxan and efaroxan is that, in addition to its potent alpha2-antagonist properties, the latter also has significant agonist activity at 5-HT1A (5-hydroxytryptamine; serotonin) receptors [24]. We, therefore, investigated whether the ability of idazoxan to cause beta-cell death was subject to modulation by the selective 5-HT1A antagonist, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine (NAN-190). However, this agent failed to significantly modify the decrease in MTS reduction caused by exposure of BRIN-BD11 cells to idazoxan (results not presented).

In view of these data and the recent results of Zaitsev et al. [19] and Papaccio et al. [21], efaroxan was selected for further investigation as a potentially protective agent that might
reduce the extent of beta-cell death in response to the cytokine IL-1beta. Treatment of BRIN-BD11 cells with IL-1beta resulted in a large increase in medium nitrite accumulation (Figure 6) consistent with the expected induction of an isoform of nitric oxide synthase and an increase in the generation of NO under these conditions. Efaroxan alone did not lead to enhanced NO production and it failed to attenuate the response to IL-1beta (Figure 6). As expected, efaroxan also had no effect on the inducible nitric oxide synthase (iNOS) independent production of NO arising from the use of a chemical NO donor, S-nitrosoglutathione (GSNO; Figure 6).

Consistent with the increased NO production, treatment of BRIN-BD11 cells with IL-1beta was associated with a significant reduction in cell viability, as judged by MTS reduction (Figure 2). Efaroxan failed to antagonise the loss of viability mediated by IL-1beta. These results were confirmed by FACS® analysis of cellular DNA (Figure 4) which revealed that, whereas efaroxan failed to alter the integrity of cellular DNA, a large increase in DNA fragmentation resulted from exposure of the cells to IL-1beta (Figure 4). The simultaneous presence of efaroxan did not attenuate this response but, surprisingly, caused a further increase in the extent of DNA fragmentation. Thus, these studies confirm that efaroxan does not directly promote BRIN-BD11 cell death and reveal that this compound fails to protect these cells from the cytotoxic effects of IL-1beta. Indeed, efaroxan appears to enhance the sensitivity of the cells to this cytokine such that the extent of DNA damage induced by interleukin-1beta was increased in cells exposed to both efaroxan and interleukin-1beta.

**DISCUSSION**

Compounds with an imidazoline structure are showing increasing promise for use in the management of type 2 diabetes since certain members of this class (e.g. efaroxan) have the capacity to stimulate insulin secretion in a strictly glucose-dependent manner [1, 2, 3, 4, 5]. However, recent data have indicated that efaroxan may also have a second important functional property since, at least in ob/ob mouse islets, it has been reported to antagonise the induction of apoptosis mediated by IL-1beta [19]. A second imidazoline, RX871024, also exerted similar effects in isolated islets [19, 21]. As a consequence, it has been suggested that imidazoline compounds may have the potential to slow the progress of beta-cell loss in type 1 diabetes, since IL-1beta-mediated toxicity is thought to play an important role in this disease.

The antagonism of IL-1beta responses by RX871024 in mouse and rat islets was reported to be due to blockade of the induction of iNOS and was associated with a significant reduction in medium nitrite accumulation when islet cells were exposed to both IL-1beta and the drug [19, 21]. It was assumed that this response occurred primarily in beta-cells but the possible involvement of other cell types was not formally excluded. In the present work, we have employed the pancreatic beta-cell line BRIN-BD11 to study these responses further since these cells respond to both IL-1beta and imidazolines and they represent a homogeneous population of clonal beta-cells.
Unlike the situation in ob/ob mouse islets [19], efaroxan failed to attenuate either the increase in medium nitrite induced by IL-1beta or the induction of DNA damage in BRIN-BD11 cells. Indeed, in these cells, efaroxan was found to 'enhance' the extent of DNA damage in IL-1beta-treated cells. Thus, it appears that the protective effects of efaroxan on IL-1beta-induced iNOS induction and NO production seen in ob/ob mouse islets are not reproduced in BRIN-BD11 beta-cells (nor in RAW macrophages, HIT-T15 or RINm5F cells; Gao H, Chan SLF, Morgan NG; unpublished observations). Since these various beta-cell types do respond to efaroxan with changes in insulin secretion [17, 23, 25, 26], it follows that the mechanisms by which efaroxan controls secretion are likely to be regulated independently of any actions on cell viability. Indeed, the possibility remains that the protective effects of imidazolines reported in islets, could be mediated by an indirect mechanism that does not derive from an interaction of the drugs with the beta-cells themselves. If so, this would not deny the potential importance of the response but it would confirm that the primary target is separate from the imidazoline binding site involved in control of insulin secretion.

Of particular significance is the present finding that, in BRIN-BD11 cells, efaroxan enhanced the DNA-damaging effects of interleukin-1beta. This was not due to any increase in NO production when the two agents were combined (Figure 6) but provides evidence that, although efaroxan was not directly cytotoxic, it sensitised the cells to the damaging effects of interleukin-1beta. It is unclear why this effect of imidazolines has not been seen in normal islet cells [19, 21] but it is unlikely to be due to any difference in the imidazoline binding site regulating insulin secretion since efaroxan increases secretion in both normal islets and in BRIN-BD11 cells [17, 23]. The mechanisms involved in this enhancing effect now warrant further study in order to investigate whether pancreatic beta-cells can be sensitised to cytotoxic insults by exposure to imidazolines under other circumstances, including during in vivo administration. If so, then their utility as potential anti-diabetic agents will require additional scrutiny.

It is important to emphasise that, despite its enhancing effect on the response to interleukin-1beta, efaroxan did not exert any acute or chronic beta-cell toxicity per se [18, 19]. This result accords with the finding that the number of apoptotic cells is not increased in ob/ob mouse islets during culture with efaroxan in vitro [19]. However, these results contrast markedly with our observation that a close structural analogue of efaroxan, idazoxan, causes a dramatic loss of viability in BRIN-BD11 cells. The extent of this response was similar to that seen previously in RINm5F and HIT-T15 cells [18] and the results suggest that pancreatic beta-cells exhibit a differential sensitivity to idazoxan and efaroxan.

Examination of the profile of cellular DNA in idazoxan-treated beta-cells by FACS® analysis, revealed that the drug induced a large increase in DNA fragmentation within only a few hours of exposure. This would be consistent with the possibility that idazoxan caused the early entry of the cells into apoptosis. In support of this, an increased number of BRIN-BD11 cells showed positive surface staining with annexin-V (a marker for the plasma membrane phosphatidylserine translocation occurring as an early step in the apoptotic pathway) after exposure to idazoxan for 12 h (Gao H, Morgan NG; unpublished data).

The molecular mechanism by which idazoxan induces beta-cell death has not been disclosed but we observed that there was no increase in medium nitrite accumulation after exposure of BRIN-BD11 cells to the drug. This implies that, unlike the situation with IL-1beta [22], idazoxan-toxicity does not involve an increase in iNOS activity or NO generation. In addition, we have also observed that the effects of idazoxan were not prevented by co-incubation with either alpha2-adrenoceptor ligands (including efaroxan [18]) or with a specific antagonist of 5-HT1A receptors. Thus, neither the potent alpha2-antagonist activity nor the 5-HT1A agonist properties of the agent
[24] can account for its cytotoxicity. In addition, on the basis of earlier observations [18] the ability of idazoxan to bind to I1- and I2-imidazoline sites can also be excluded. Thus, idazoxan appears to initiate the rapid, apoptotic, demise of pancreatic beta-cells by a mechanism that does not involve binding to any of the receptor sites currently defined as targets for this ligand. This response is not restricted to clonal beta-cell lines since morphological evidence indicates that the cells of normal islets are also subject to damage when exposed to idazoxan in culture [20].

Taken together, the present results confirm that structurally related imidazoline drugs can exert markedly different effects on the viability of pancreatic beta-cells. They do not provide support for the view that efaroxan can attenuate the cytotoxic actions of IL-1beta in a pure population of clonal beta-cells but reveal that efaroxan enhances the DNA-damaging effects of the cytokine in BRIN-BD11 cells. Since BRIN-BD11 cells express an efaroxan-sensitive protein involved in control of insulin secretion [17, 23] these results imply that binding of ligands to this protein does not lead to attenuation of IL-1-induced NO formation.

In view of the fact that some imidazoline reagents are being actively studied as potential anti-diabetic drugs [1, 2, 3, 4, 5] and that others are already in clinical use as centrally-acting anti-hypertensive agents [2, 15] and as alpha2-adrenoceptor antagonists [4, 15], it is important that their effects on beta-cell viability are defined in greater detail. Such studies may also lead to an improved understanding of the pathways involved in regulating the entry of pancreatic beta-cells into apoptosis.

Keywords Apoptosis; Biological Phenomena, Cell Phenomena, and Immunity; Cell Death; Cell Physiology; Chemicals and Drugs Category; Dioxanes; Dioxins; Growth Substances; Heterocyclic Compounds; Heterocyclic Compounds, 1-Ring; Idazoxan; Imidazoles; Interleukin-1

Abbreviations 5-HT: (5-hydroxytryptamine, serotonin; FACS®: fluorescence activated cell sorting; IL: interleukin; GSNO: S-nitrosoglutathione; iNOS: inducible nitric oxide synthase; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; NAN-190: 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine; PBS: phosphate buffered saline; PMS: phenozine methosulphate; RPMI: Roswell Park Memorial Institute

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References
1. Morgan NG, Chan SL. Imidazoline binding sites in the endocrine pancreas: can they fulfil their potential as targets for the development of new insulin secretagogues? Curr Pharm Des 2001; 7:1413-31. [PMID 11472276]


