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Short communication

Cytotoxic action of methylglyoxal on insulin-secreting cells

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Abstract

Methylglyoxal is a spontaneous product of glucose metabolism which is known to have cytotoxic actions and to be present in raised concentrations in hyperglycaemia. It could therefore play an important role in glucose toxicity. We have investigated the cytotoxic effects of methylglyoxal on insulin-secreting cells, which are particularly sensitive to glucose toxicity. Methylglyoxal caused a concentration-dependent increase in the number of apoptotic RINm5F cells within 4–6 hours. A similar effect was observed with rat pancreatic β-cells. *tert*-butylglyoxal, which is a poor substrate for the glyoxalase pathway, exerted a similar, though more potent apoptotic action. Dexamethasone and NaF were also found to induce apoptosis in RINm5F cells. Flow cytometric analysis suggested a degree of necrosis in addition to apoptosis resulting from treatment with methylglyoxal. The cytotoxic effect of methylglyoxal could contribute towards glucose toxicity in insulin-secreting cells.

Keywords: RINm5F cell; Pancreatic β-cell; Apoptosis; Methylglyoxal; Glucose toxicity

1. Introduction

The accelerated loss of pancreatic β-cells in IDDM is thought to occur, at least in part, by a process of apoptotic cell death [1,2]. Inflammatory cytokines produced in the islet in IDDM, including interleukin-1β, interferon γ and tumour necrosis factor α, have been shown not only to impair β-cell function but to induce apoptosis [3–5].

In addition to the role of cytokines in β-cell apoptosis, it is possible that raised concentrations of glucose, characteristic of diabetes mellitus, could also be a contributory factor. The phenomenon of glucose toxicity can affect a wide range of cell types, and may play a major role in the development of diabetic secondary complications [6]. In particular, chronic hyperglycaemia is known to result in pancreatic β-cell dysfunction [7]. Furthermore, hyperglycaemia-induced β-cell apoptosis has been demonstrated *in vivo* in two animal models of NIDDM; [8,9] and also *in vitro* [10,11]. The molecular mechanisms underlying glucose toxicity appear to be complex, and may involve glycation of cellular proteins and oxidative stress [11] and possibly, at least in the case of β-cells, raised levels of $[Ca^{2+}]_i$ [10].

Prolonged hyperglycemia is associated with raised levels of methylglyoxal [12], a spontaneous by-product of glycolysis which interacts strongly with cellular proteins and nucleic acids and has pronounced cytotoxic activity (see ref. [13] for review). We have demonstrated that methylglyoxal exerts marked acute effects, including depolarization and raised $[Ca^{2+}]_i$, in insulin-secreting cells [14]. Recent studies have also demonstrated that methylglyoxal causes apoptotic cell death in macrophage-derived cell lines [15] and in leukemic T- and 60 cells [16,17]. The present study therefore investigated the cytotoxic actions of methylglyoxal on insulin-secreting cells.

2. Materials and methods

RINm5F cells were maintained in continuous culture in RPMI medium supplemented with 10% foetal bovine serum, 50 U/mL penicillin, and 50 U/mL streptomycin (all from Gibco, Paisley, UK). Cells were grown in an atmosphere of 95% O_2/5% CO_2 at 37° and were used between passage numbers 60 and 75. Cells were passaged before reaching confluency using trypsin-EDTA (Gibco). RINm5F

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Abbreviations: Ho 33342, Hoechst 33342 dye; IDDM, insulin-dependent diabetes mellitus; NIDDM, non insulin-dependent diabetes mellitus; $[Ca^{2+}]_i$, cytosolic $[Ca^{2+}]$; PI, propidium iodide.

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cells are derived from a rat insulinoma and show many similarities to native rat pancreatic β-cells and, being an homogeneous cell population, are highly suitable for this type of work. However, these cells are poorly-responsive to glucose. Therefore, a number of experiments were carried out using rat pancreatic β-cells, prepared essentially as described previously [18], except that dispersal of islets into single cells was facilitated by a 5 min. incubation with trypsin (0.25% w/v). In both cases, cells were plated onto glass coverslips and placed in 24 well plastic dishes (NUNC) in 800 μL of culture medium containing appropriate test agents. At given time points, 10 μL Ho33342 was added to each well to give a final concentration of 10 μM and the cells incubated for a further 10 min. to allow dye uptake. The coverslips were then removed from the wells, washed with RPMI and the cells viewed under a Nikon fluorescence microscope at 350 nm excitation, 450 nm emission. Cells undergoing apoptosis were identified from their fragmented nuclei. A population of 100 cells was viewed and, using two cell counters, the numbers of apoptotic and normal cells counted, the number of apoptotic cells being expressed as a percentage of the total cell population.

Apoptotic cells were also identified using multiparameter flow cytometry as previously described [19]. Briefly, a single-cell suspension of RINm5F cells was prepared by triturating through a 1-mL syringe. These cells were then incubated at room temperature with Ho 33342 (10 μM) for 5 min. Just before analysis, 30 μM PI was added and 10,000 cells were analyzed with respect to forward and orthogonal light scatter together with red (PI-DNA, linear scale) and blue (Ho 33342-DNA, log scale) fluorescence at a flow rate of 300–400 cells per second using a FACS Vantage flow cytometer (Becton-Dickinson).

Ho 33342, and PI were purchased from Molecular Probes. Methylglyoxal and *tert*-butylglyoxal were synthesized as described previously [20,21]. All other chemicals were obtained from Sigma. Statistical significance was ascribed using Student's *t*-test.

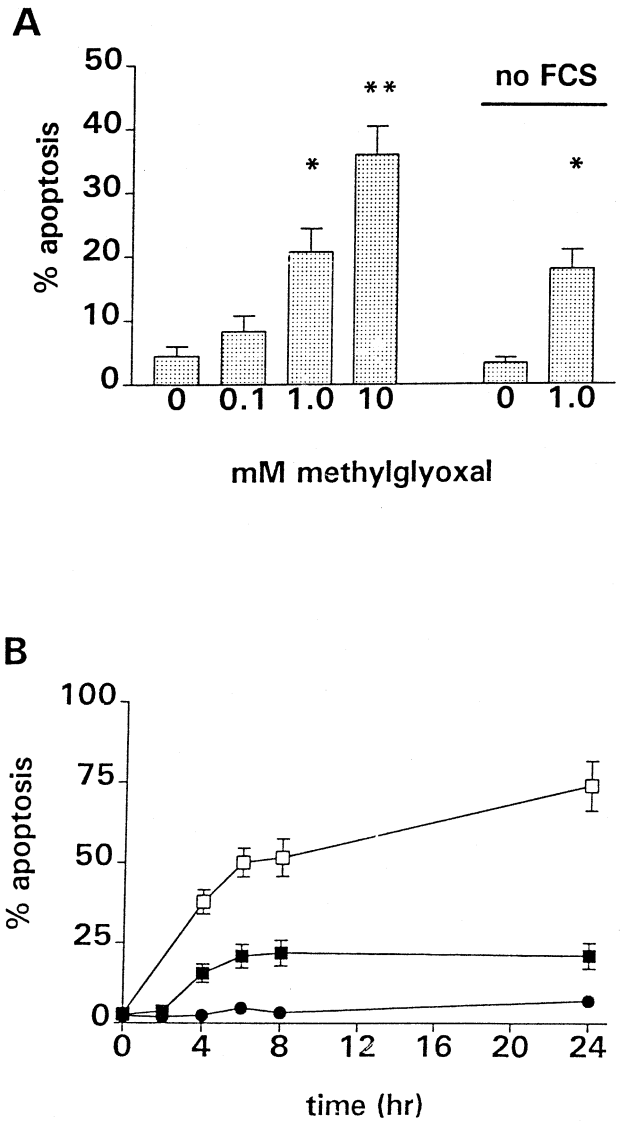

Fig. 1. (A) Induction of apoptosis in RINm5F insulin-secreting cells by methylglyoxal in the presence or absence of foetal calf serum (FCS; 5% v/v). Cells were cultured in the absence or presence of methylglyoxal for 6 hr. All values are mean ± SEM of 5–6 determinations. *$P < 0.01$; **$P < 0.001$. (B) Time courses of effects of 1 mM methylglyoxal (■) and 1 mM *tert*-butylglyoxal (□) on apoptosis in RINm5F cells compared to control (●). Each point is the mean ± SEM of 8–14 determinations.

3. Results and discussion

Culture of RINm5F cells for 6 hours in the presence of methylglyoxal increased the percentage of apoptotic cells in a concentration-dependent manner within the range 0.1–10 mM (Fig. 1A). This concentration range exceeds the levels of free methylglyoxal found *in vivo*, even under pathological conditions (human plasma levels ~100–500 nM [12, 22]). However, since methylglyoxal is formed during glycolysis [13], it is likely that the intracellular concentrations of methylglyoxal are considerably higher than circulating levels [23]. In this respect, pancreatic β-cells have a relatively high capacity for glycolysis [24], and it is therefore likely that they will be exposed to particularly high levels of methylglyoxal during hyperglycaemia.

The ability of methylglyoxal to induce apoptosis in RINm5F cells was independent of the presence of foetal calf serum (Fig. 1A), indicating that the cytotoxic action of methylglyoxal was not affected by its binding to serum proteins. It is possible that this was due to dissociation of methylglyoxal from serum protein complexes or due to apoptotic actions of the methylglyoxal-protein adducts which are known to be biologically active [25]. A significant ($P < 0.01$) effect of methylglyoxal (1 mM) on apoptosis was detectable within 4 hours, although at this concentration, the maximum percentage of apoptotic cells was reached at 6 hours (Fig. 1B). In common with most, if not all mammalian cells, insulin-secreting cells have an active

glyoxalase pathway which converts methylglyoxal to D-lactate [13,14]. In order to assess the possible importance of this pathway in the cytotoxic action of methylglyoxal, we next used *tert*-butylglyoxal which is a poor substrate for glyoxalase I [26]. As shown in Fig. 1B, the percentage of apoptotic cells when exposed to 1 mM *tert*-butylglyoxal was significantly greater ($P < 0.01$ or less) at all time points compared to cells cultured with an equivalent concentration of methylglyoxal. It is likely that the inability of the cells to detoxify *tert*-butylglyoxal could contribute towards the increased number of apoptotic cells, although it is also possible that the reactivity of *tert*-butylglyoxal with nucleotides and proteins is different from that of methylglyoxal. Since *tert*-butylglyoxal does not share the ability of methylglyoxal to depolarize and increase $[Ca^{2+}]_i$ in insulin-secreting cells [14], this finding also suggests that the rise in $[Ca^{2+}]_i$ evoked by methylglyoxal plays little or no role in inducing apoptosis.

We compared the ability of methylglyoxal to induce apoptosis with that of other agents previously reported to cause apoptosis in insulin-secreting cells and other cell types. Exposure to dexamethasone (10 μM), a drug previously shown to induce apoptosis in CEM-C7A lymphoblastoid cells [27], was found to cause apoptosis in RINm5F cells to a degree comparable to that induced by 1 mM methylglyoxal (Fig. 2A and B). As previously reported [28], application of NaF (5 mM) also caused a marked stimulation of apoptosis in RINm5F cells, exceeding 50% at 6 hr (Fig. 2A). It has been suggested that this effect indicates a central role of heterotrimeric G-proteins in the modulation of apoptotic cell death [28].

In order to confirm the microscopy data, we also examined RINm5F cells treated with methylglyoxal for 24 hr using multiparameter flow cytometry. This method allows the simultaneous analysis of chromatin condensation and plasma membrane integrity. In this assay apoptotic cells are smaller and stain more brightly with the fluorophore Ho 33342. Furthermore, a second fluorophore, PI, will only stain the nuclei of cells which have lost membrane integrity [19]. A contour plot of Ho 33342 fluorescence and forward light scatter (which is proportional to cell size) allows normal and apoptotic cells to be distinguished (Fig. 3). It is clear from this figure that treatment of RINm5F cells with methylglyoxal for 24 hr increases the number of events in the apoptotic population ("population A"; Figs. 3A and C). However, if the number of events staining positive for PI are also included, then many of the cells which have been designated apoptotic have also lost membrane integrity (Figs. 3B and D). These data are summarized in Fig. 4 and show that the number of RINm5F cells that are apoptotic at 24 hr (27.8% ± 5.4%), has only increased slightly from the number obtained at 8 hr (20.8% ± 3.7%) using fluorescence microscopy. However, the proportion of apoptotic cells that have lost their membrane integrity is 73.3% ± 5.7%. This proportion of membrane "leaky" cells is larger than what is usually observed with this assay because apoptotic cells do

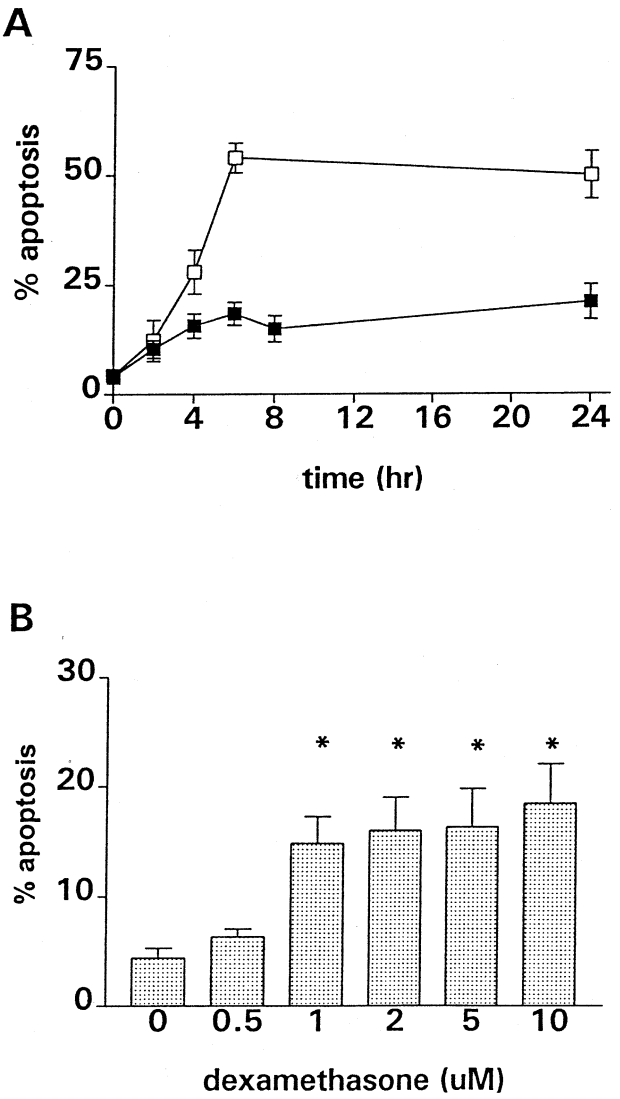

Fig. 2. (A) Time courses of effects of 10 μM dexamethasone (■) and 5 mM NaF (□) on apoptosis in RINm5F cells. Each point is the mean ± SEM of 4–7 determinations. (B) Effect of increasing concentrations of dexamethasone on apoptosis in RINm5F cells. Cells were cultured in the absence or presence of dexamethasone for 6 hr. All values are mean ± SEM of 4–6 determinations. *$P < 0.01$.

not normally lose their membrane integrity until they fragment; these fragments being commonly referred to as secondary necrosis [19]. Therefore, these data suggest that while methylglyoxal is inducing the nuclear changes associated with apoptosis, its toxicity may be such that it also damages the cell membrane at an earlier stage than would normally be observed. As such methylglyoxal toxicity may be borderline to producing a more chaotic necrotic cell death.

The extent of apoptosis was examined in rat pancreatic β-cells cultured for 40 hours at different glucose concentrations. In the absence of glucose, the percentage of apoptotic cells was 50.2 ± 4.1 (N = 19). When glucose was added at concentrations of 5 or 11 mM, the corresponding values for apoptosis were significantly reduced to 24.2% ± 3.1% (N = 18; $P < 0.001$) and 14.6% ± 2.0% (N = 20;

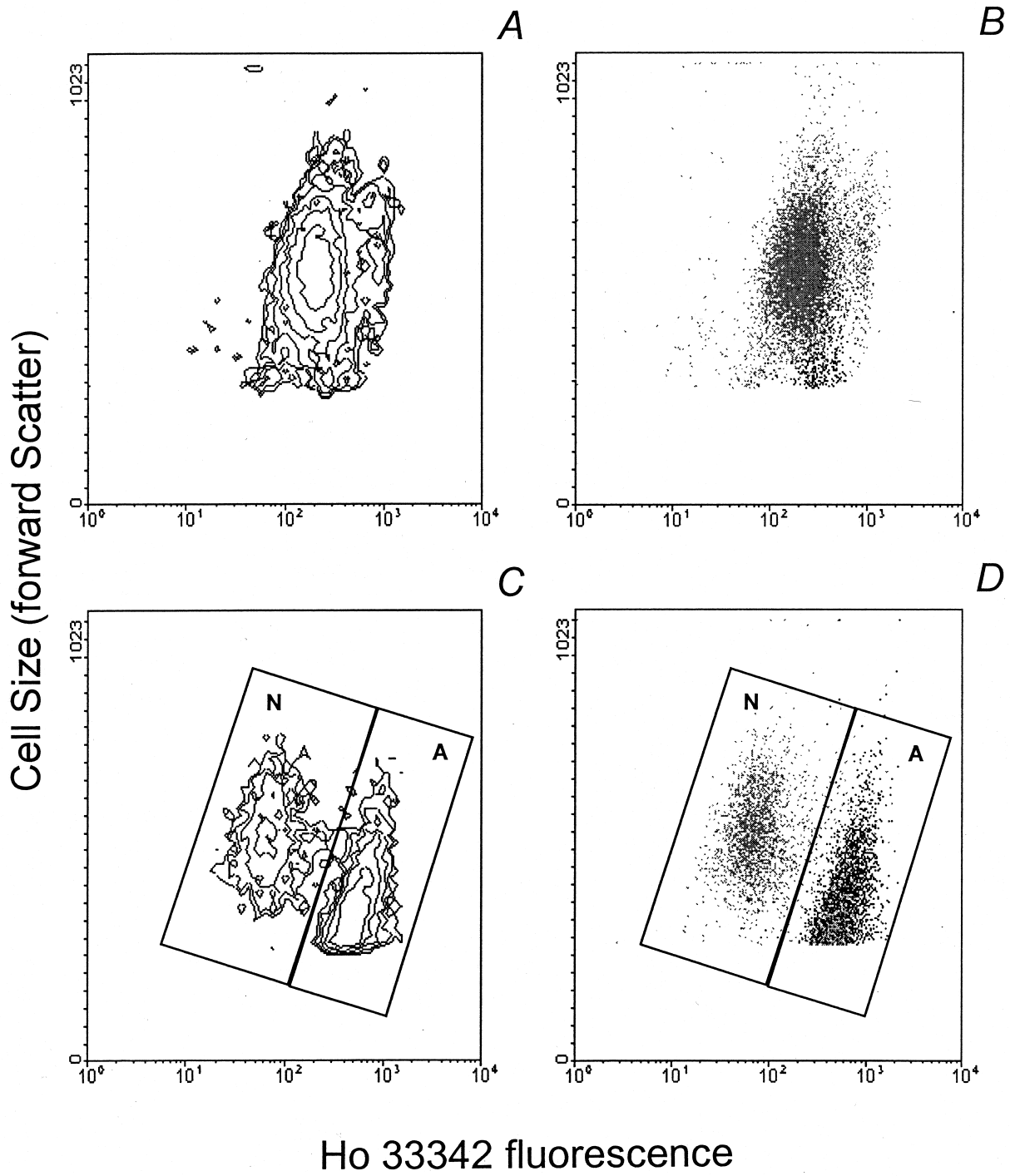

Fig. 3. Multiparameter flow cytometric analysis of RINm5F cells treated for 24 hr with 1 mM methylglyoxal (C and D) or vehicle control (A and B). RINm5F cells were prepared for flow cytometry and stained with Ho 33342 and PI as described in section 2. The data are expressed as both a contour (A and C) or a dot plot (B and D). On the dot plots, cells which have lost membrane integrity and so stain positive for PI are coded black while the remainder are rendered grey. Apoptotic cells (population **A**) were distinguished from normal cells (population **N**) by their increased Ho 33342 fluorescence and their decreased forward light scatter. Events with a forward light scatter of less than 267 units have been excluded from this analysis. These plots are representative of 15 other experiments.

$P < 0.001$). In the presence of 16 mM glucose, the percentage of apoptotic cells was 40.3 ± 4.4 (N = 16), a value significantly greater ($P < 0.001$) than that in the presence of 11 mM glucose. This biphasic effect of glucose on β-cell apoptosis is consistent with previous reports suggesting that glucose can exert both a protective action at intermediate concentrations (~10 mM; [29]) and also a cytotoxic action at higher concentrations [10]. It is possible that, in glucose-starved β-cells, the intracellular concentration of GSH falls [30] leading to increased susceptibility to oxidative stress and

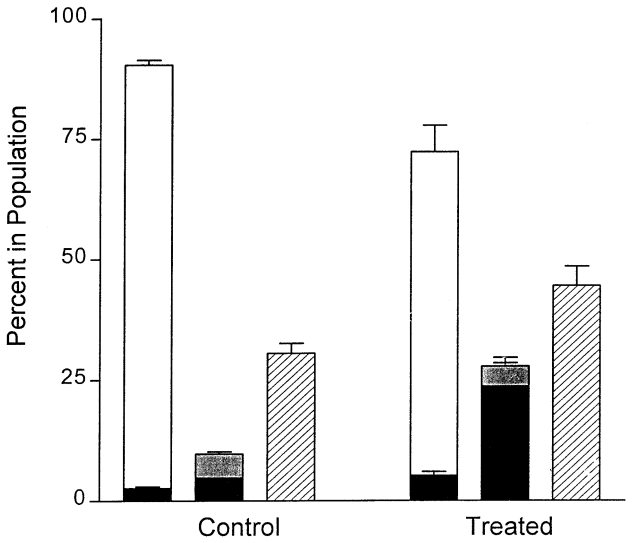

Fig. 4. The percentage of events with low Ho 33342—normal forward scatter (*open bars*) that correspond to normal cells (population **N** in Fig. 3), high Ho 33342—decreased forward scatter (stippled bars) that correspond to apoptotic cells (population A in Fig. 3), and events that have been excluded from analysis because their forward scatter is below 267 units (diagonal lined bars). The percentage of events that were positive for PI in the normal and apoptotic subpopulations are indicated by the filled bars. Each bar represents the mean ± SEM of 16 experiments.

cytotoxicity [31]. This could, at least in part, explain the protective effects of glucose at intermediate concentrations. On the other hand, the cytotoxic action of high concentrations of glucose could be due to prolonged elevation of $[Ca^{2+}]_i$ [10], glycation reactions [11] and possibly other factors.

When rat β-cells were cultured for 40 hr in the presence of 11 mM glucose and 1 mM methylglyoxal, the percentage of apoptotic cells was significantly increased to 64.3 ± 3.6 ($N = 18$; $P < 0.001$). The increment in apoptotic cells attributable to methylglyoxal was considerable greater in the case of rat pancreatic β-cells (approx. 50%) than with RINm5F cells (approx. 14%), indicating that the insulinoma cell line may be less susceptible than native β-cells to the cytotoxic action of methylglyoxal, although we cannot at present provide any explanation for this finding.

In conclusion, methylglyoxal exerts a cytotoxic action on RINm5F insulinoma cells, and on rat pancreatic β-cells. This effect is probably due to interaction with and consequent modification of cellular proteins and nucleotides, and could contribute towards cellular damage and apoptosis known to result from prolonged exposure to high concentrations of glucose. It should, however, be borne in mind that the relationship between the concentrations of methylglyoxal which cause cytotoxicity and intracellular levels of the α-ketoaldehyde is at present unclear.

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