Apoptosis (2006) 11:1231–1238
DOI 10.1007/s10495-006-7450-7

Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β-cells exposed to palmitate

Hannah J. Welters · Eleftheria Diakogiannaki · J. Mark Mordue · Moh Tadayyon · Stephen A. Smith · Noel G. Morgan

Published online: 11 May 2006

Abstract Saturated and mono-unsaturated fatty acids exert differential effects on pancreatic β-cell viability during chronic exposure. Long chain saturated molecules (e.g. palmitate) are cytotoxic to β-cells and this is associated with caspase activation and induction of apoptosis. By contrast, mono-unsaturated fatty acids (e.g. palmitoleate) are not toxic and can protect against the detrimental effects of palmitate. In the present study, we show that the protective actions of palmitoleate in BRIN-BD11 β-cells result in attenuated caspase activation following exposure to palmitate and that a similar response occurs in cells having elevated levels of cAMP. However, unlike palmitoleate, elevation of cAMP was unable to prevent the cytotoxic actions of palmitate since it caused a diversion of the pathway of cell death from apoptosis to necrosis. Palmitoleate did not alter cAMP levels in BRIN-BD11 cells and the results suggest that a change in cAMP is not involved in mediating the protective effects of this fatty acid. Moreover, they reveal that attenuated caspase activation does not always correlate with altered cell viability in cultured β-cells and suggest that mono-unsaturated fatty acids control cell viability by regulating a different step in the apoptotic pathway from that influenced by cAMP.

Keywords Islets of Langerhans · BRIN-BD11 cells · Palmitoleate · Mono-unsaturated fatty acid · Apoptosis · Forskolin

H. J. Welters · E. Diakogiannaki · J. M. Mordue · N. G. Morgan (✉)
Institute of Biomedical and Clinical Science,
Peninsula Medical School,
Devon, Research Way, Plymouth PL6 8BU, UK
e-mail: noel.morgan@pms.ac.uk

M. Tadayyon
Boehringer-Ingelheim Pharma,
Biberach, Germany

S. A. Smith
GlaxoSmithKline Pharmaceuticals,
Harlow, Essex, UK

Introduction

It has become increasingly evident from studies in both animal models and in man, that type 1 and type 2 diabetes are each associated with a net loss of β-cells [1–5]. The molecular mechanisms remain to be fully determined but appear to involve the inappropriate activation of apoptosis in both cases. Since this is not compensated by a corresponding increase in the rate of β-cell replacement, the net result is a gradual decline in β-cell numbers. This occurs early in the development of type 1 diabetes and somewhat later in type 2 but, nevertheless, β-cell apoptosis plays a significant role in the progression of each form of the disease [1–5]. As a consequence, it is clear that interventions that are designed to protect β-cells from pro-apoptotic events will have important therapeutic potential and may also provide novel insights into the molecular mechanisms involved in the regulation of apoptosis.

A variety of agents can attenuate β-cell apoptosis *in vitro* and *in vivo* [6–12] and, among the most effective, are a series of monounsaturated fatty acids, including palmitoleate and oleate [13–16]. These fatty acids exert potent protective effects against both lipotoxic insults and cytokine-mediated β-cell death [13] suggesting that they regulate a critical anti-apoptotic pathway. In addition, certain incretin hormones, including glucagon-like peptide 1 (GLP-1) [10, 12] and glucose-dependent insulinotropic polypeptide (GIP)

[17, 18] are also powerfully anti-apoptotic in β-cells although it is not known whether similar mechanisms are responsible for the effects of incretins and monounsaturated fatty acids. In the case of GLP-1, it has been proposed that the anti-apoptotic activity may be related to an increase in cAMP in β-cells [11, 19, 20] and a rise in cAMP was shown to cause the inhibition of caspase-3 activity when these cells were exposed to the saturated fatty acid, palmitate [11]. This was associated with a small improvement in cell viability although the blockade of cell death was incomplete.

On the basis of this evidence, it seems possible that a rise in cAMP may serve to inhibit caspase activation and β-cell apoptosis under lipotoxic conditions [11, 19]. However, it is not known whether the ability of monounsaturated fatty acids to protect against the cytotoxic activity of the saturated fatty acid, palmitate, is accompanied by a rise in β-cell cAMP. It is important to evaluate this possibility since pancreatic β-cells express the fatty acid-responsive cell surface receptor, GPR40 [21–23], and this has recently been proposed to be capable of coupling to Gs in β-cells (thereby raising cAMP) [24]. Therefore, in the present study, we have compared the effects of a pharmacological elevation of cAMP with the protective effects of palmitoleate in cells exposed to palmitate, to establish whether a rise in cAMP can be implicated in mediating the anti-apoptotic response to the mono-unsaturated fatty acid.

Materials and methods

Cell culture

The rat pancreatic β-cell line BRIN-BD11 was selected for the present studies as the regulation of its viability by saturated and mono-unsaturated fatty acids is similar to that seen with human β-cells [13, 14]. The cells were cultured in RPMI-1640 medium containing 11 mM glucose, 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C. Cells were grown and maintained in 75 cm^2 flasks and used for individual experiments when they had reached 80% confluence.

Exposure of BRIN-BD11 cells to fatty acids

The fatty acid palmitate (Sigma) was initially dissolved in ethanol: water (1:1, v:v) by heating to 70°C. Palmitoleate (ICN) was dissolved in 90% ethanol at room temperature. The fatty acids were then bound to bovine serum albumin by mixing with a 10% solution of fatty acid free bovine albumin (Sigma) at 37°C for 1 h. For individual experiments, fatty acids were added to modified RPMI (containing 5.5 mM glucose) to yield 0.5% ethanol, 1% BSA and appropriate concentrations of the fatty acid. BRIN-BD11 cells were treated with these fatty acid mixtures, in the presence or absence of other test compounds, 24 h after seeding into 6 well plates (1×10^5 cells/well). Control cells received ethanol and BSA alone.

Vital dye staining

To determine the proportion of cell death in BRIN-BD11 cells, floating and attached cells were collected, centrifuged at 350 g for 5 min, and resuspended in 250 μl of complete RPMI 1640 medium. They were then mixed with an equal volume of Trypan Blue (0.4% in PBS) and the cells counted using a haemocytometer. The percentage of dead cells was calculated for each experimental condition.

Apoptosis assays

CaspACE™ FITC-VAD-FMK In Situ Marker (Promega) is a fluoroisothiocyanate conjugate of the cell permeable caspase inhibitor VAD-FMK. Following exposure to fatty acids, cells were harvested and resuspended at a density of 7.5×10^6 cells/ml. 10 μl was removed and mixed with 10 μl of CaspACE substrate (5 μM final concentration). Cells were incubated in the dark at 37°C for 20 min, then centrifuged at 1000 g for 5 min and washed in 200 μl PBS. The final cell pellet was resuspended in 50 μl PBS, transferred to poly-L-lysine coated microscope slides and incubated at room temperature for 5 min before viewing under a fluorescent microscope.

In some studies, the extent of apoptosis and cell necrosis was compared directly using a combination of Hoechst 33342 and propidium iodide to distinguish live, apoptotic and necrotic cells under fluorescence illumination. Cells were stained with a mixture of propidium iodide (5 μg/ml) and Hoechst 33342 (50 μg/ml). The staining mixture was added to the cell media for 5 min and protected from light. The cells were then viewed using a fluorescent microscope. Hoechst 33342 can enter all cells and stains the DNA blue. Viable cells thus appear pale blue, whereas apoptotic cells have bright blue condensed nuclei. Necrotic cells with compromised membranes also take up propidium iodide and stain pink.

cAMP measurement

Intracellular cAMP was measured with the cAMP Biotrack Enzymeimmunoassay system (Amersham Bioscience) using the non-acetylation procedure. BRIN-BD11 cells were seeded in 12 well plates at 2×10^5 cells/well, 24 h before addition of test compounds. After exposure, cells were lysed in 250 μl of lysis buffer and the enzyme immunoassay carried out according to the manufacturer's instructions.

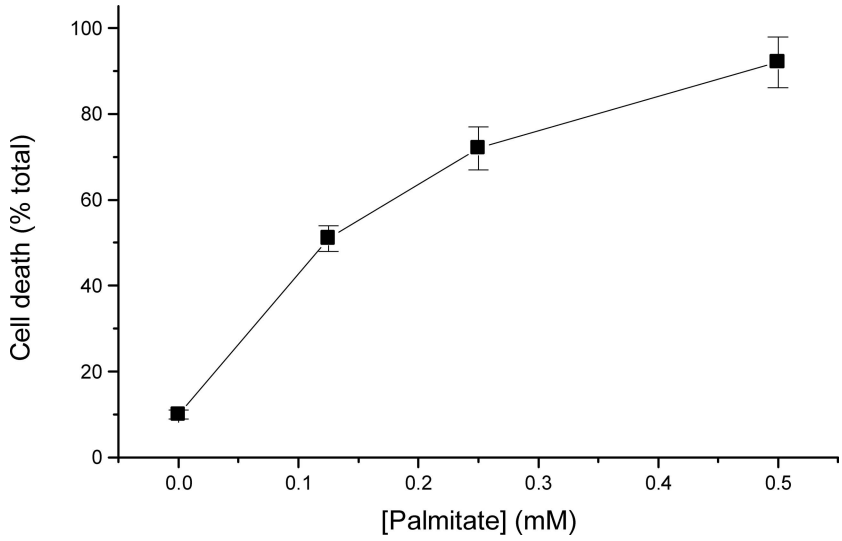

Fig. 1 Effects of palmitate on β-cell viability. BRIN-BD11 cells were treated with increasing concentration of palmitate for 18 h and the extent of cell death estimated by vital dye staining. A significant loss of viability was seen with all palmitate concentrations tested ($p < 0.001$). Data are mean values ± SEM ($N = 4$)

Statistical analysis

All individual experiments were performed in at least duplicate and were repeated on a minimum of 3 separate occasions. The results were analysed by ANOVA and were considered significant when $p < 0.05$.

Results

Since, in previous studies [11, 12, 25] it has been established that the saturated fatty acid, palmitate (C16:0), causes an increase in β-cell death when cells are cultured in the presence of this agent, palmitate was selected as the lipotoxic stimulus in the present experiments. As shown in Fig. 1, incubation of BRIN-BD11 cells with increasing concentrations of palmitate (0.1–0.5 mM) resulted in a dose-dependent loss of viability. This effect occurred in a time-dependent manner (not shown) and was due, at least in part, to an increase in apoptosis since it was significantly attenuated by the broad-specificity, cell permeable, caspase inhibitor, Z-VAD-fmk (Fig. 2). In confirmation of this, large numbers of apoptotic cells could be identified after exposure of BRIN-BD11 cells to palmitate as judged by various methods including treatment with a fluorescent caspase substrate and after staining with Hoechst 33342 and propidium iodide (see below).

To examine further the induction of cell death caused by exposure to palmitate, the overall level of cell viability and the activation of caspases were compared in parallel in cells exposed to 0.25 mM palmitate (Fig. 3). As expected, palmitate caused a significant decrease in total cell viability over an 18 h period and this was accompanied by increased caspase activation (Fig. 3) consistent with induction of apoptosis. In marked contrast to palmitate, the corresponding mono-unsaturated fatty acid, palmitoleate (C16:1; 0.25 mM) failed to induce cell death and caused no activation of caspases during 18 h of incubation with BRIN-BD11 cells (Fig. 3). Palmitoleate was equally well-tolerated during

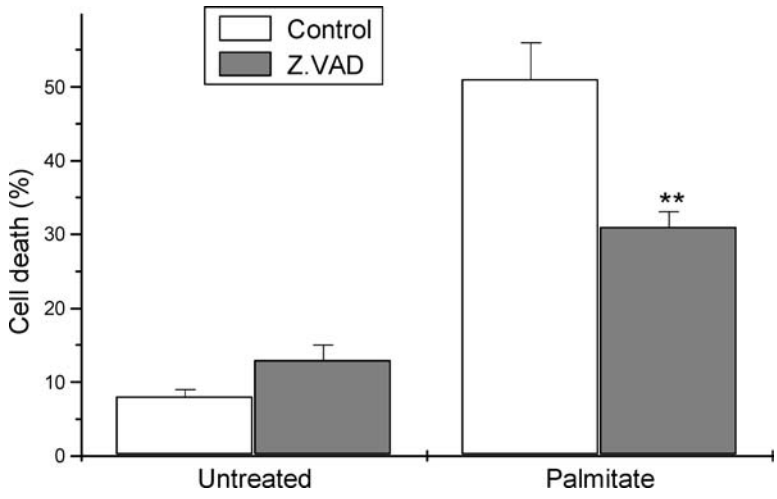

Fig. 2 Effects of the caspase inhibitor Z-VAD-fmk on palmitate-induced cell death. BRIN-BD11 cells were treated with 0.25 mM palmitate for 18 h in the absence or presence of the broad specificity caspase inhibitor, Z-VAD-fmk (100 μM), as shown. The extent of cell death estimated by vital dye staining. Data are mean values ± SEM from 3 experiments.$^{**}p < 0.001$ vs palmitate alone

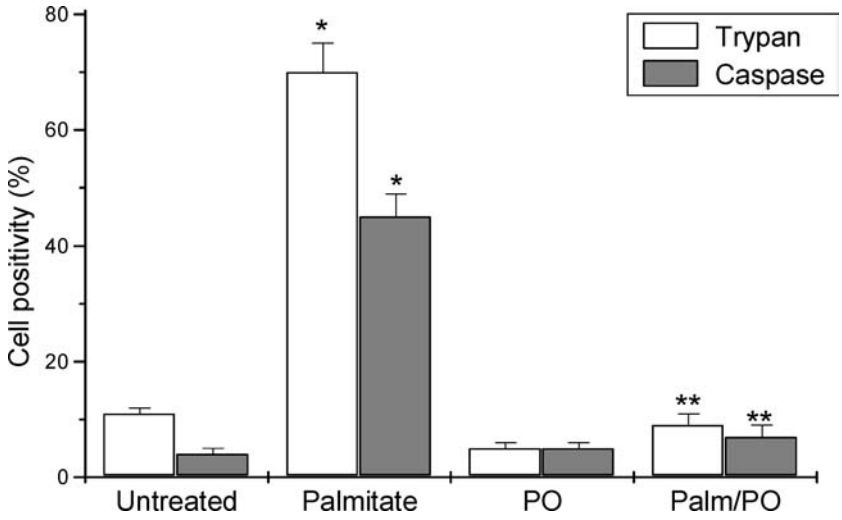

Fig. 3 Effects of fatty acids on cell viability and caspase activation in BRIN-BD11 cells. BRIN-BD11 cells were treated with 0.25 mM palmitate and/or 0.25 mM palmitoleate (PO) as shown, for 18 h. After this time, the extent of cell death was estimated by vital dye staining and the level of caspase activation assessed by fluorescence microscopy after exposure of the cells to CaspACE™ FITC-VAD-fmk. Data are mean values ± SEM from 3 experiments. $^{*}p < 0.001$ vs untreated cells; $^{**}p < 0.001$ vs palmitate alone

longer periods of incubation since no loss of viability above control values was observed even after 72 h exposure to this agent (cell death in control: 1.6 ± 0.1%; 0.25 mM palmitoleate: 1.3 ± 0.1%). When added together with 0.25 mM palmitate, palmitoleate (0.25 mM) completely prevented the loss of viability caused by the saturated fatty acid and it also abolished the induction of caspase activity seen under these conditions (Fig. 3).

The blockade of apoptosis by palmitoleate is reminiscent of that reported previously when pancreatic β-cells were exposed to palmitate in the presence of agents that promote a rise in cAMP [11]. Under these circumstances, palmitate-mediated caspase activation was attenuated by the increase in cAMP and therefore, the present studies were extended to examine whether a rise in cAMP could underlie the attenuation of apoptosis mediated by palmitoleate. Accordingly, cAMP levels were measured in BRIN-BD11 cells during incubation with fatty acids. This revealed that neither 0.25 mM palmitate nor 0.25 mM palmitoleate caused any alteration in β-cell cAMP (Fig. 4). By contrast, the combination of a phosphodiesterase inhibitor, IBMX (0.1 mM), and the adenylate cyclase activator, forskolin (10 μM) provoked a large rise in cAMP (Fig. 4).

In view of this clear difference between the effects of IBMX/forskolin and palmitoleate on cAMP levels in BRIN-BD11 cells, we felt it important to reconsider whether the combination of IBMX/forskolin could attenuate palmitate-induced caspase activation in β-cells. Therefore, the cells were cultured with 0.25 mM palmitate in the absence and presence of IBMX/forskolin for 18 h and both caspase activation and the overall extent of cell death were measured in parallel (Fig. 5). As expected, palmitate provoked a rise in caspase activation (measured fluorescently) and in total cell death (estimated with trypan blue) and, in agreement with a previous study [11], caspase activation was markedly attenuated in cells exposed to IBMX/forskolin (Fig. 5). However, most importantly (and unlike the situation with palmitoleate) the inhibition of caspase activation seen in the presence of IBMX/forskolin, was not accompanied by any overall improvement in cell viability. Rather, the ability

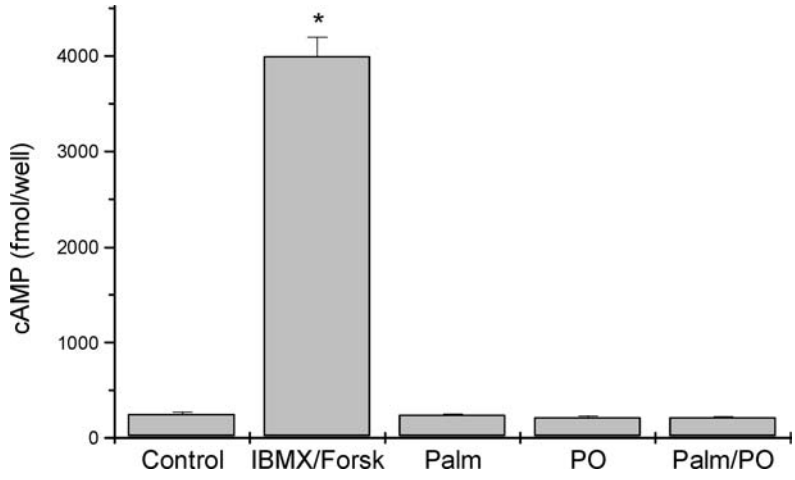

Fig. 4 cAMP levels in BRIN-BD11 cells following exposure to fatty acids. BRIN-BD11 cells were exposed to 0.25 mM palmitate, 0.25 mM palmitoleate (PO) or a combination of IBMX (100 μM) and forskolin (10 μM) for 6 h and the levels of cAMP measured by ELISA. Data are mean values ± SEM ($N = 6$). $^{*}p < 0.001$ vs control cells

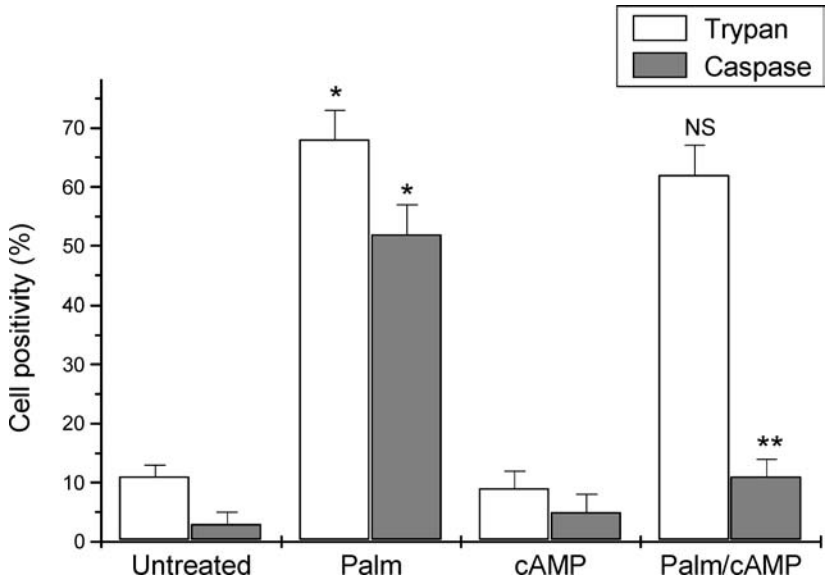

Fig. 5 Figure 3 effects of an elevation of cAMP on cell viability and caspase activation in BRIN-BD11 cells treated with palmitate. BRIN-BD11 cells were treated with 0.25 mM palmitate in the absence or presence of IBMX (100 μM) and forskolin (10 μM) [cAMP] for 18 h. After this time, the extent of cell death was estimated by vital dye staining and the level of caspase activation assessed by fluorescence microscopy after exposure of the cells to CaspACE^{TM} FITC-VAD-fmk. Data are mean values ± SEM from 3 experiments. $^{*}p < 0.001$ vs untreated cells; $^{**}p < 0.001$ vs palmitate alone; NS—not significant

of the saturated fatty acid to kill β-cells was unaffected under these conditions. Thus, there was a clear dissociation between the ability of IBMX/forskolin to attenuate caspase activation and to regulate cell viability in palmitate-treated β-cells. By contrast, the monounsaturated fatty acid palmitoleate, lowered caspase activation and also prevented the loss of cell viability. Moreover, both of these responses occurred in the absence of a measurable rise in cAMP.

To address these issues further, additional experiments were performed in which the mode of cell death in palmitate-treated cells (whether necrosis or apoptosis) was investigated by selective staining with Hoechst 33342 and propidium iodide. These dyes can be used to distinguish live, necrotic and apoptotic cells under fluorescence illumination such that the proportions of each can be estimated. For these studies, 100 μM palmitate was employed to minimise the possibility that the number of necrotic cells might be over-estimated (due to development of the features of secondary necrosis after an initial apoptotic mode of death).

As expected, when BRIN-BD11 cells were cultured under control conditions (presence of 1% BSA but no added fatty acid) the cells were mainly viable and relatively few apoptotic or necrotic cells were present (Fig. 6). Incubation in the presence of palmitate (100 μM) resulted in the appearance of numerous apoptotic cells while the increase in necrotic cells was much smaller. This confirms that apoptosis was the principal mode of cell death under these conditions. Elevation of cAMP with IBMX/forskolin failed to prevent palmitate-induced cell death (in accord with the vital dye staining shown in Fig. 5) but, under these conditions, there was a dramatic alteration in the numbers of apoptotic vs necrotic cells in the population. When cAMP was elevated with IBMX/forskolin, the number of apoptotic cells was markedly reduced but there was a corresponding rise in cell necrosis (Fig. 6*)*. Thus, under these conditions, it appeared that the elevation of cAMP had induced a switch from a primarily apoptotic mode of cell death (with palmitate alone) to a largely necrotic mode of death (palmitate in the presence of elevated cAMP; Fig. 6), however, the rise in cAMP was not able to attenuate the loss of viability caused by palmitate.

Discussion

This results of this study have confirmed that there are dramatic differences between the effects of saturated and mono-unsaturated long chain free fatty acids on pancreatic β-cell viability [13–16]. Thus, the saturated C16 fatty acid, palmitate, caused a net loss of β-cell viability whereas the C16:1 mono-unsaturate, palmitoleate, was not toxic to the cells. The cytotoxic effect of palmitate was dose-dependent and occurred within the expected physiological range of total palmitate concentrations (Fig. 1). Indeed, under the conditions of these experiments (i.e. with bovine serum albumin present in the culture medium) the free concentration of palmitate was probably in the sub-μM range [1, 26], suggesting that the cells are poorly tolerant of palmitate when it is present as the sole fatty acid. Thus, it can be concluded that, in common with several other cell types [27–30], pancreatic

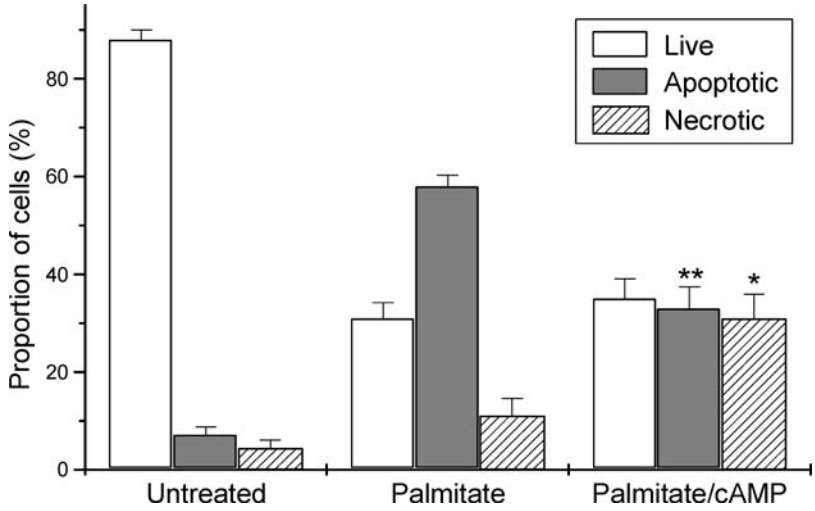

Fig. 6 Levels of apoptosis and necrosis in BRIN-BD11 cells exposed to palmitate in the absence or presence of cAMP. BRIN-BD11 cells were treated with 0.1 mM palmitate in the absence or presence of IBMX (100 μM) and forskolin (10 μM) [cAMP] for 18 h. After this time, the mode of cell death was assessed by fluorescence microscopy after staining of cells with propidium iodide and Hoecsht 33342. Numbers of live, apoptotic and necrotic cells were recorded. Data are mean values ± SEM obtained in 5 experiments. $^{*}p < 0.005$ vs necrosis in the presence of palmitate alone; $^{**}p < 0.005$ vs apoptosis in the presence of palmitate alone

β-cells are susceptible to palmitate-induced cytotoxicity and that this effect occurs at relatively low concentrations of the fatty acid.

The contrasting effects of the saturated and mono-unsaturated C16 free fatty acids on cell viability were striking but, equally remarkable, was the finding that the cytotoxic effect of palmitate is modulated by the availability of the latter. This protective action against β-cell cytotoxicity is reminiscent of that exerted by certain incretins (e.g. GLP-1 and GIP) which have been suggested to mediate their effects by increasing the level of cAMP in β-cells [11, 19, 20]. On this basis, it has been proposed that cAMP may propagate an anti-apoptotic signal in β-cells and, in support of this, it was recently reported that elevation of cAMP leads to inhibition of palmitate-induced caspase activation in β-cells [11]. This would be consistent with a mechanism by which cAMP regulates the entry of β-cells into apoptosis and raises the important possibility that mono-unsaturated fatty acids might also mediate their anti-apoptotic effects by increasing the level of cAMP. This is particularly true in the light of recent evidence that β-cells express the cell surface receptor GPR40, which is activated by certain fatty acids and may mediate some of the effects of these agents on β-cell secretory function [21–24]. Under most conditions, it appears that GPR40 preferentially couples to Gq and promotes changes in intracellular Ca^{2+} handling but a recent report has suggested that this receptor may also couple to Gs, leading to altered cAMP levels in β-cells exposed to fatty acids [24]. Therefore, it is important to establish whether the protective effects of palmitoleate involve a rise in cAMP since such a mechanism might implicate signalling via a Gs-mediated pathway in response to activation of GPR40.

When BRIN-BD11 cells were exposed to palmitoleate under conditions known to promote the preservation of cell viability, cAMP levels were not increased (Fig. 4). Palmitate also failed to alter cAMP in the cells. Therefore, it seems unlikely that a rise in cAMP underlies the ability of palmitoleate to protect the cells against palmitate-induced cytotoxicity. This conclusion does not formally exclude the possibility that activation of GPR40 might be involved in mediating the response to palmitoleate but the selectivity of binding of fatty acids to this receptor also makes this unlikely. For example, both long chain saturated fatty acids and long chain mono-unsaturated fatty acids are reported to be agonistic at GPR40 and this pharmacology does not accord with their differential effects on viability seen here [21, 22]. Thus, the present results imply that a rise in cAMP deriving from the activation of GPR40 is unlikely to account for the protective effects of palmitoleate against β-cell cytotoxicity.

The conclusion that a rise in cAMP is not involved in mediating the protective effects of palmitoleate does not necessarily imply that cAMP cannot exert anti-apoptotic effects in β-cells. Indeed, we have confirmed the findings of Kwon et al. [11] that a rise in cAMP leads to attenuated caspase activation in cells exposed to palmitate. However, our results suggest that cAMP-dependent blockade of caspase activation is not necessarily sufficient to protect the cells from the cytotoxic actions of palmitate. Rather, we show that, in the case of β-cells exposed to palmitate, these two parameters are clearly separable under certain conditions. Most strikingly, we found that in cells treated with IBMX/forskolin to elevate cAMP, palmitate-induced caspase activation was inhibited very markedly but the total level of cell viability was not improved in a corresponding manner. Rather, overall

viability was still low but the proportion of cells exhibiting the features of apoptosis had declined in favour of those displaying the characteristics of necrosis. This situation contrasts with that observed in cells exposed to palmitate and palmitoleate, when viability was maintained at a very high level and the loss of apoptotic cells was not accompanied by any increase in the number of cells dying by necrosis.

The present finding that inhibition of caspase activation can be dissociated from protection against loss of viability in β-cells exposed to palmitate, extends the findings of El-Assaad et al. [31] who reached similar conclusions using INS-1 cells treated with Z-VAD-fmk. In the present work, we found that Z-VAD-fmk attenuated palmitate-induced cell death in BRIN-BD11 cells (Fig. 2) suggesting that INS-1 cells and BRIN-BD11 cells may respond differently to this agent. Nevertheless, the results of both studies support the view that distal control mechanisms exist in β-cells that can regulate the switch between apoptosis and necrosis.

Conclusion

The present study reveals that a rise in cAMP can divert the cell death pathway from apoptosis to necrosis in fatty acid-treated β-cells. The molecular mechanism has not been disclosed but the present observations demonstrate that this diversion can occur under conditions when caspase activation is attenuated. Hence, they demonstrate that measurements of caspase activation are not always a reliable indicator of the extent of cell viability when β-cells are incubated *in vitro*. The results also show that mono-unsaturated fatty acids can prevent the diversion of apoptosis to necrosis under conditions when caspase activation is inhibited which suggests that they regulate a critical component of the cell death pathway which is different from that controlled by cAMP. Identification and selective targeting of this pathway may provide significant benefit as a means to improve β-cell viability during culture of islets prior to transplantation since, at present, significant losses occur during islet isolation and culture *in vitro*.

Acknowledgments We are grateful to Diabetes UK, Northcott Devon Foundation and the GB Sasakawa Foundation for financial support.

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