Mono-unsaturated fatty acids protect against β-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure

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Abstract Long-chain saturated fatty acids are cytotoxic to pancreatic β-cells while shorter-chain saturated and long-chain unsaturated molecules are better tolerated. Mono-unsaturated fatty acids are not, however, inert since they inhibit the pro-apoptotic effects of saturated molecules. In the present work we show that the mono-unsaturates palmitoleate (C16:1) or oleate (C18:1) also cause marked inhibition of apoptosis induced by exposure of clonal BRIN-BD11 β-cells to serum withdrawal or a combination of interleukin-1β plus interferon-γ. This response was dose-dependent and not accompanied by changes in NO formation. Taken together, the results suggest that mono-unsaturated fatty acids regulate a distal step common to several apoptotic pathways in pancreatic β-cells.

Key words: β-Cell; Lipotoxicity; Apoptosis; Diabetes; Interleukin-1β; Palmitoleate

1. Introduction

Loss of pancreatic β-cells by increased apoptosis is now recognised as a feature common to both type 1 and type 2 diabetes [1,2], although the mechanisms responsible are likely to be different in each case. In type I diabetes, an autoimmune reaction is mounted against specific pancreatic islet antigens leading to infiltration of islets by macrophages and T-cells [3]. These cells then secrete cytokines (such as interleukin-1β (IL-1β), interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα)) which induce the transcription of nitric oxide synthase (iNOS) and cause the production of NO in the β-cells [3–5]. Moderate elevation of NO then results in the activation of apoptosis [6] although higher levels may also cause necrotic death [7]. By contrast, in type 2 diabetes, a still undefined process of 'lipotoxicity' is responsible for β-cell death [8]. This also results from an increase in apoptosis and it is believed that elevated circulating saturated free fatty acids (FFA) are primarily responsible [9–11], although their effects are probably exacerbated by accompanying hyperglycaemia [8,12,13]. Increased production of NO has been reported upon exposure of islets to FFA [14–16] but other evidence suggests that fatty acid-mediated β-cell toxicity is independent of NO formation [17–19].

Despite the evidence that β-cell lipotoxicity is mediated by circulating FFA, it has also become clear that fatty acids differ markedly in their ability to promote β-cell apoptosis. Long-chain saturated molecules appear to be the most cytotoxic while the equivalent mono-unsaturates are ineffective and have even been shown to protect β-cells against the toxicity of saturated fatty acids [18,20–24]. The reasons for this difference have not been established although they could relate to differential partitioning of the various species into neutral lipids [18,25]. An attractive alternative hypothesis is that the differences may relate to the ability of unsaturated molecules to preferentially stabilise mitochondria, thereby minimising cytochrome *c* release and caspase activation [26,27]. In support of this, Maedler et al. [20] have recently shown that unsaturated fatty acids can attenuate mitochondrial cytochrome *c* release in β-cells.

If this hypothesis is valid and mitochondrial stabilisation is important for the protective effects of unsaturated fatty acids, then it might be expected that these molecules would also improve the viability of pancreatic β-cells exposed to other cytotoxic stimuli (in addition to saturated fatty acids). This is because the mitochondrial events represent a common distal component involved in the activation of apoptosis by multiple stimuli [28]. However, to date, the effects of unsaturated fatty acids on other inducers of apoptosis have not been systematically tested although, in one study in insulin-secreting INS-1 cells, it was observed that oleate enhanced the response to IL-1β [29]. Therefore, in the present work, we have examined the effects of selected saturated and unsaturated fatty acids on pancreatic β-cell viability. In particular, we have investigated whether long-chain mono-unsaturates differentially regulate apoptosis induced by either lipid (saturated fatty acids) or non-lipid (serum withdrawal; cytokines) stimuli.

2. Materials and methods

2.1. Materials

All fatty acids except palmitoleate (from ICN) were purchased from Sigma. Most cell culture reagents were purchased from Invitrogen, although foetal bovine serum was from PPA Laboratories. Etomoxir was a kind gift from Dr Greg Cooney. IL-1β and IFN-γ were pur-

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Abbreviations: FFA, free fatty acid; iNOS, inducible nitric oxide synthase; IFNγ, interferon-γ; IL-1β, interleukin-1β

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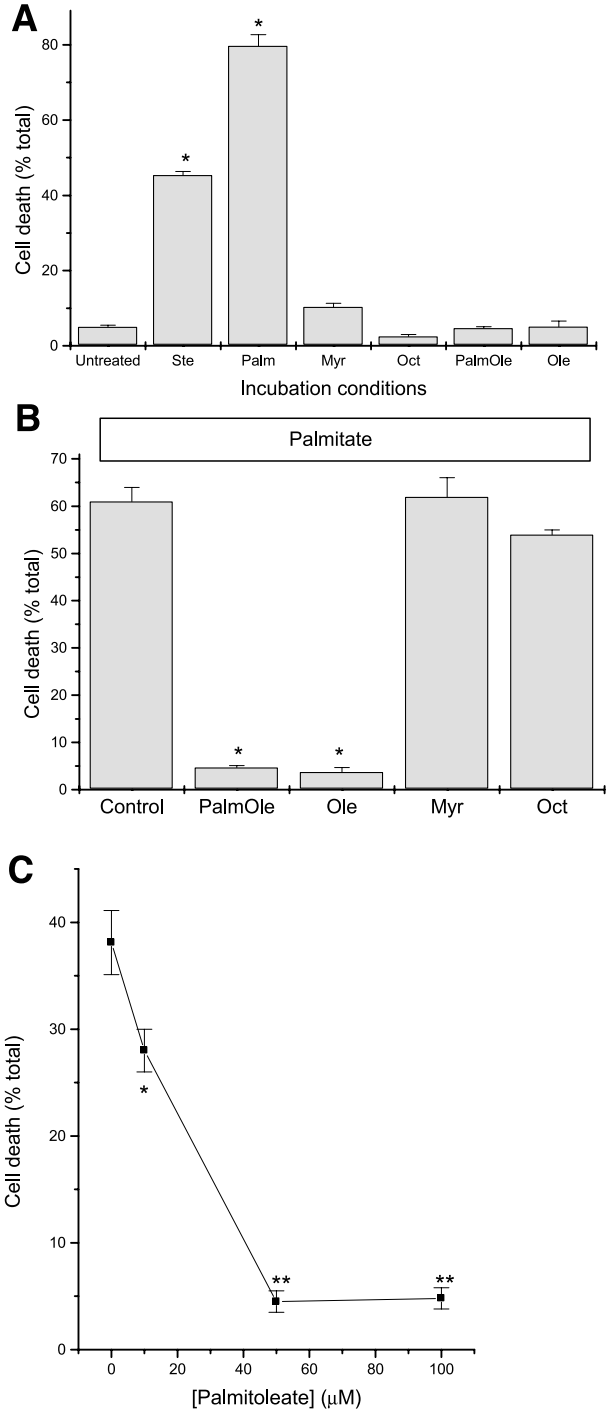
chased from Calbiochem. All other reagents were purchased from Sigma.

2.2. Cell culture

The insulin-secreting rat β-cell line BRIN-BD11 [30] was 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. Cells were cultured at 37°C in 5% CO_2 and were used in experiments when approximately 80% confluent.

2.3. Preparation of fatty acids and treatment of cells

Fatty acid solutions for use in cell incubations were prepared in accordance with Jacqueminet et al. [31]. Stearate, palmitate, myristate and octanoate were each initially dissolved in 50% ethanol by heating to 70°C. Oleate and palmitoleate were dissolved in 90% ethanol. The dissolved fatty acids were then bound to albumin by mixing with a 10% fatty acid-free bovine albumin solution at 37°C for 1 h. This mixture was added to modified RPMI 1640 medium containing 5.5 mM glucose to give final concentrations of 0.5% ethanol and 1% bovine serum albumin (BSA). Cells were treated with the fatty acid mixtures plus appropriate compounds in 6 well plates seeded the previous day with 1×10^5 cells/well. All control wells received vehicle alone (0.5% ethanol and 1% BSA).



2.4. Measurement of cell death

For routine determination of cell death, vital dye staining was used. Floating and attached cells were collected from each well, centrifuged at $200\times g$ for 5 min and resuspended in 150 μl of RPMI 1640 medium. This was then mixed with 150 μl of trypan blue (0.4% in phosphate-buffered saline (PBS)). The numbers of live and dead cells were counted using a haemocytometer and the percentage dead cells calculated.

In some experiments, induction of apoptosis was measured using either annexin V-Cy3 staining or measurements of caspase activation.

2.4.1. CaspACE assay. 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 in 50 μl of PBS. 10 μl was removed and mixed with 10 μl of CaspACE substrate (10 μM final concentration). Cells were incubated in the dark at 37°C for 20 min, then centrifuged at $1000\times g$ 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.

2.4.2. Annexin V-CY3 staining. The annexin V-Cy3 apoptosis detection kit (Sigma) employs two labels: annexin-Cy3.18, a red fluorescent protein, which binds to phosphatidylserine present in the outer leaflet of the plasma membrane of cells undergoing apoptosis, and 6-carboxyfluorescein diacetate (6-CFDA). 6-CFDA measures cell viability since it is hydrolysed by esterases present in living cells to produce the green fluorescent compound 6-carboxyfluorescein (6-CF). Hence, these two labels can distinguish between live, necrotic and apoptotic cells. BRIN-BD11 cells were washed twice then resuspended in PBS at a density of 1×10^6 cells/ml. The cell suspension (50 μl) was added to poly-L-lysine-coated slides which were washed three times in binding buffer (Sigma) before addition of 50 μl of staining solution (containing AnnCy3 and 6-CFDA). The cells were incubated for 10 min at room temperature in the dark, before observation under a fluorescent microscope.

2.5. Nitrite assay

Nitrite formation was measured as an indicator of NO production. Cells were incubated in a total volume of 1 ml with test reagents for 24 h. After this time samples of medium were removed for nitrite measurement by the Greiss assay [6]. Samples were incubated with a mixture (1:1 v:v) 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.

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Fig. 1. Effects of fatty acids of varying chain length and degree of saturation on cell death in BRIN-BD11 cells. A: Fatty acids (stearate (Ste), palmitate (Palm), myristate (Myr), octanoate (Oct), palmitoleate (PalmOle), oleate (Ole) – all at 0.5 mM) were bound to albumin then introduced into the incubation medium of BRIN cells for a period of 18 h. After this time the cells were harvested and their viability measured by vital dye staining. $^*P<0.001$ relative to untreated control cells. B: BRIN cells were exposed to 0.5 mM palmitate for 18 h in the absence (control) or presence of palmitoleate (PalmOle; 0.5 mM), oleate (Ole; 0.5 mM), myristate (Myr; 0.5 mM) or octanoate (Oct; 0.5 mM). After this time the cells were harvested and their viability measured by vital dye staining. $^*P<0.001$ relative to cells exposed to palmitate alone. C: BRIN cells were treated with palmitate (0.5 mM) in the absence or presence of increasing concentrations of palmitoleate as shown. After incubation for 18 h, the cells were harvested and their viability measured by vital dye staining. $^*P<0.05$, $^{**}P<0.001$ relative to palmitate alone.

2.6. Statistical analysis

All individual experiments were performed at least in duplicate and were repeated on a minimum of three separate occasions. The results were analysed by analysis of variance with Tukey's test and were considered significant when $P < 0.05$.

3. Results

3.1. Comparative effects of fatty acids on β-cell viability

Initially, the effects of a range of saturated and unsaturated fatty acids were compared on the viability of BRIN-BD11 β-cells (Fig. 1). The results confirmed that the cells display a marked difference in sensitivity to these agents, with long-chain saturated molecules (palmitate (C16:0) and stearate (C18:0) up to 0.5 mM) causing a large increase in cell death while the equivalent unsaturated molecules (palmitoleate (C16:1) and oleate (C18:1) up to 0.5 mM) were well tolerated (Fig. 1A). Saturated fatty acids having a chain length shorter than C16 (e.g. myristate (C14:0) or octanoate (C8:0)) were also ineffective as inducers of cell death (Fig. 1A). The cell death caused by palmitate was largely mediated by increased apoptosis as it was accompanied by caspase activation and increased annexin V-Cy3 staining ($2.3 \pm 0.6\%$ apoptotic cells in control vs $30.6 \pm 3.5\%$ after 18 h exposure to 0.1 mM palmitate; $P < 0.001$).

An important difference between the effects of shorter-chain saturated fatty acids and long-chain mono-unsaturates was observed when cells were exposed to combinations of FFA (Fig. 1B). Whereas myristate and octanoate failed to influence the extent of cell death caused by exposure of BRIN cells to palmitate (or stearate – not shown), palmitoleate and oleate dramatically attenuated the cytotoxic effects of the saturated molecules. This response was dose-dependent (Fig. 1C) and concentrations of palmitoleate as low as 10 μM caused a significant improvement in viability compared to cells that were exposed to palmitate (500 μM) in the absence of palmitoleate. The effects of palmitoleate did not result from any alteration

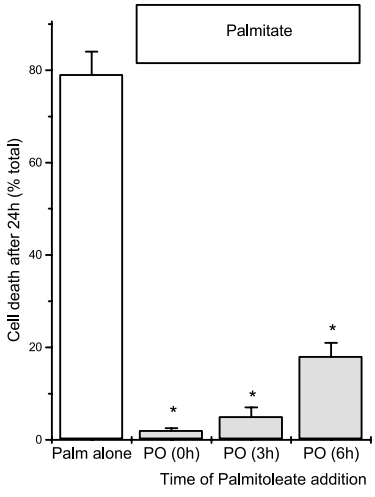

Fig. 2. Time dependence of the antagonism of palmitate-induced toxicity by palmitoleate. BRIN cells were treated with 0.5 mM palmitate (Palm) bound to 1% fatty acid-free albumin. After 0, 3 or 6 h of incubation with palmitate, 0.25 mM palmitoleate (PO) was also added and the culture period continued. The extent of cell death was determined after a total of 24 h culture for all incubation conditions. *$P < 0.001$ relative to palmitate alone.

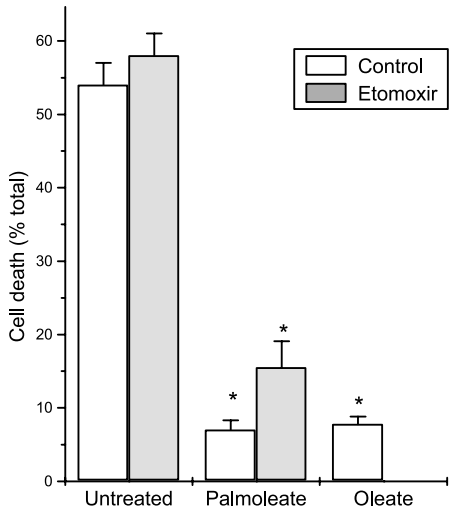

Fig. 3. Effects of unsaturated fatty acids on the increase in BRIN-BD11 cell death induced by serum withdrawal. BRIN cells were cultured in RPMI 1640 medium lacking added serum but containing fatty acid-free albumin. Palmitoleate (0.5 mM) or oleate (0.5 mM) was included as shown and some cells also received etomoxir (0.2 mM; grey bars). Cells were harvested after culture for 48 h and their viability determined. *$P < 0.001$ relative to untreated cells.

in the rate of cell proliferation, which was unchanged during exposure to this fatty acid over at least 72 h (not shown).

Parallel studies established that the increase in BRIN cell death induced by palmitate does not result from non-specific alterations in plasma membrane integrity but that a time-dependent change in the transcription of still unidentified genes is required (Welters et al., in preparation). Therefore, in the present studies, cells were exposed to palmitoleate at different times after the initial addition of palmitate to establish whether the mono-unsaturated molecule can attenuate apoptosis after initiation of the response to palmitate. The results revealed (Fig. 2) that the addition of palmitoleate to the cells up to 6 h after palmitate was still effective at blocking the increased apoptosis normally seen in response to palmitate.

3.2. Effects of mono-unsaturated fatty acids on β-cell death induced by serum withdrawal or cytokine exposure

The results described above imply that palmitoleate attenuates β-cell apoptosis by exerting an influence at a relatively late step in the apoptotic pathway. On this basis, we considered the possibility that, if this step is also required for induction of apoptosis by other agents, these might also be sensitive to inhibition by mono-unsaturated fatty acids. Thus, in the next series of experiments, BRIN cell death was induced by the removal of survival factors that accompanies serum withdrawal. As expected, culture of BRIN cells in medium devoid of serum led to a marked loss of viability over 48 h (Fig. 3). However, the extent of this response was dramatically reduced when the cells were also exposed to palmitoleate (or oleate; Fig. 3) during the period of serum withdrawal. The protective effects of palmitoleate were not prevented by the presence of the carnitine palmitoyltranferase-1 inhibitor etomoxir, confirming that mitochondrial oxidation of the fatty acid was not required for this response.

To extend these observations further, BRIN cells were next treated with a combination of cytokines (IL-1β (2 μg/μl) plus IFNγ (1 U/μl)). Exposure of the cells to these agents resulted

in a large accumulation of nitrite in the culture medium, consistent with the induction of iNOS (and the consequent increase in NO formation) expected under these conditions (Fig. 4A). Nitrite formation was not changed by co-incubation with palmitoleate implying that the fatty acid exerted no effect on iNOS induction. It was also established that neither palmitate nor palmitoleate induced the production of NO from BRIN cells (Fig. 4A). Despite its failure to inhibit cytokine-induced NO production, treatment of BRIN cells with palmitoleate caused a significant improvement in cell viability during exposure to IL-1β and IFNγ (Fig. 4B). This suggests that the mono-unsaturated fatty acid was able to attenuate the cytotoxic effects of IL-1β and IFNγ by an action that lies downstream of NO generation.

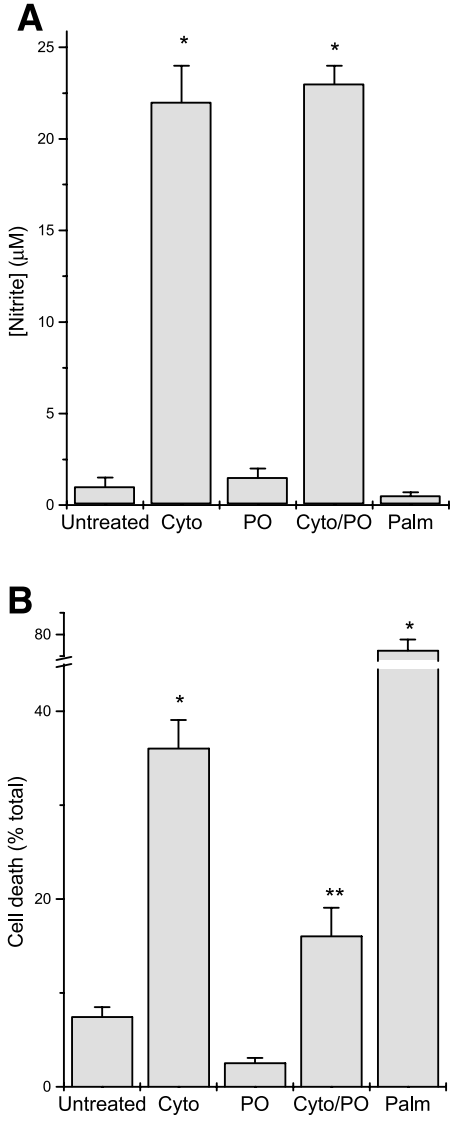

Fig. 4. Effects of fatty acids and cytokines on nitrite formation and cell death in BRIN-BD11 cells. A: Cells were left untreated or were exposed for 24 h to cytokines [IL-1β (2 pg/μl) plus IFNγ (1 U/μl)], palmitoleate (PO; 0.5 mM) or palmitate (Palm; 0.5 mM) as shown. After this time the medium was sampled and the extent of nitrite formation measured. $*P < 0.001$ relative to untreated cells. B: Cells were left untreated or were exposed for 24 h to cytokines [IL-1β (2 pg/μl) plus IFNγ (1 U/μl)], palmitoleate (PO; 0.5 mM) or palmitate (Palm; 0.5 mM) as shown. After this time the cells were harvested and their viability measured. $*P < 0.001$ relative to untreated cells; $**P < 0.01$ relative to cells exposed to cytokines in the absence of palmitoleate.

4. Discussion

The present data demonstrate very forcibly that saturated and unsaturated fatty acids exert markedly different effects on β-cell viability. As such, they are in accordance with recent results obtained in primary islet cells [20,23] and show that cultured BRIN-BD11 cells represent a suitable model for study of the mechanisms of β-cell lipotoxicity. They also emphasise that the pro- and anti-apoptotic effects of different fatty acid species are not restricted to the non-β-cells present in islets, since the current studies were undertaken with a clonal β-cell line.

The studies have revealed that, in order to promote cell death, β-cells must be exposed to saturated fatty acids whose acyl chain is at least 16 carbon atoms in length (e.g. palmitate, C16:0). Shorter-chain molecules (such as myristate (C14:0) and octanoate (C8:0)) were well tolerated by the cells, even when present at concentrations in the mM range. This is significant since palmitate is the most abundant circulating fatty acid in vivo [32] and suggests that this molecule could mediate an increase in β-cell death under conditions when FFA levels are elevated (e.g. during the progression towards type 2 diabetes). In drawing this conclusion it is important to emphasise that we have not directly determined the free concentrations of fatty acid generated under our experimental conditions, nor have we ascertained whether cellular lipid pools are differentially affected by saturated or unsaturated fatty acids. Nevertheless, the fatty acids were complexed to albumin at ratios that can be expected to yield free fatty acid concentrations that lie within the physiological (nM) range [18,25].

The presence of one double bond in the hydrocarbon chain of a C16 or C18 fatty acid (to yield palmitoleate (C16:1) and oleate (C18:1) respectively) was sufficient to markedly attenuate the cytotoxic potential suggesting that β-cell apoptosis is initiated in a highly selective manner by only a small subset of fatty acids. Even more strikingly, it was observed that palmitoleate and oleate were able to attenuate the cytotoxic actions of palmitate (and stearate) suggesting that the unsaturated molecules exert a profoundly anti-apoptotic response in β-cells. These data are consistent with recent results in isolated human islets [20,23]. In an important extension of these studies, it was established that the effects of mono-unsaturated fatty acids were not restricted to inhibition of palmitate (or stearate)-induced toxicity since similar inhibition of cell death was also evident when either serum withdrawal or exposure to cytokines was used as the pro-apoptotic stimulus. As the underlying mechanism responsible for initiation of apoptosis in each of these circumstances is different, this implies that mono-unsaturated fatty acids must influence a distal component of the apoptotic pathway that is common to multiple stimuli.

The mechanisms of cytokine-induced apoptosis are arguably the best understood in pancreatic β-cells since it is widely accepted that treatment of cells with IL-1β and IFNγ leads to the induction of iNOS and, thereby, to an elevation of NO [2,3]. This in turn promotes cell death by virtue of increased protein and DNA nitrosylation, inhibition of key metabolic enzymes and increased cGMP generation [3–5,33]. Ultimately, these culminate in the opening of the mitochondrial perme-

ability transition pore, release of cytochrome *c* and activation of effector caspases [34]. The present finding that the ability of palmitoleate to promote β-cell viability was not associated with any reduction in nitrite formation during incubation with IL-1β and IFNγ reveals that mono-unsaturates do not regulate the early steps in this sequence. Moreover, they also reveal that the potentially 'toxic' effects of elevated NO generation can be antagonised successfully in β-cells, without necessarily down-regulating NO production per se.

It has been reported in some studies that saturated fatty acids promote an increase in β-cell NO formation [14–16] suggesting that generation of this radical could represent a common feature of the mechanisms of action of cytokines and FFA. However, increased iNOS transcription and NO production have not always been seen in cells exposed to saturated fatty acids [17–19] and we were unable to detect significant nitrite formation (an index of NO generation) in the medium of BRIN cells exposed to cytotoxic concentrations of palmitate. Thus, we conclude that the anti-apoptotic actions of palmitoleate are not mediated by reduced NO formation (in cells incubated either with saturated fatty acids or with cytokines) and that this molecule intervenes at a more distal step in the apoptotic pathway.

The mechanism by which serum withdrawal promotes apoptosis probably involves the removal of survival signals required for the long-term maintenance of viability. Such signals are principally mediated by a phosphatidylinositol 3-kinase (PI3K)-dependent mechanism that is attenuated upon removal of the various growth factors present in serum [35–37]. In breast cancer cells, oleate has been reported to activate PI3K [22] suggesting that regulation of this pathway might represent a target for the pro-apoptotic effects of unsaturated fatty acids in β-cells. Indeed, such a mechanism might also explain their ability to antagonise the pro-apoptotic effects of cytokines and saturated fatty acids. However, we have observed that treatment of BRIN cells with either of two PI3K inhibitors (wortmannin or LY294002) failed to reduce the protective effects of palmitoleate on apoptosis (H.J. Welters, C. Boyle and N.G. Morgan, unpublished observations). Thus, it is unlikely that the PI3K pathway is a principal target for the anti-apoptotic actions of mono-unsaturates in β-cells.

Therefore, it appears that mono-unsaturates regulate apoptosis by acting at a relatively distal step, which lies at, or beyond, the point of convergence of a variety of different pathways involved in promoting apoptosis. In support of this, we found that palmitoleate was still effective as an inhibitor of palmitate-induced cell death even when the unsaturated molecule was introduced as late as 6 h after initial exposure of cells to palmitate. Since the activation of apoptosis by palmitate is initiated within the first 2–4 h of palmitate exposure and changes in the transcription of early response genes are already complete by this time [38], this confirms that palmitoleate regulates a more distal step in the pathway.

The precise site of action of palmitoleate remains to be identified but it may be significant that mono-unsaturated fatty acids have recently been shown to attenuate the release of cytochrome *c* from β-cell mitochondria [21]. This represents a critical distal step in the apoptotic pathway and requires the earlier release of cytochrome *c* from binding sites located on the inner mitochondrial membrane. It is not known how this initial displacement of cytochrome *c* occurs but recent evidence implies that it may involve a change in the availability of cardiolipin [26,27]. Cardiolipin is a unique phospholipid that carries four acyl groups that are normally derived from unsaturated fatty acids. Thus, incubation of cells with mono-unsaturates will promote cardiolipin synthesis and, as a result, may inhibit cytochrome *c* release. This mechanism would explain why the non-toxic lipids myristate and octanoate failed to protect against the cytotoxicity of longer-chain saturated molecules, since neither would be expected to maintain cardiolipin levels as effectively as the mono-unsaturates. Regulation of cardiolipin synthesis could also account for the present finding that low concentrations of palmitoleate significantly inhibited cell death induced by a 50-fold greater concentration of palmitate. This is because the substrate specificity of cardiolipin synthase for unsaturated fatty acids is sufficiently high that even low concentrations would be expected to compete effectively with saturated molecules and thereby promote cardiolipin synthesis [27]. Thus, changes in cardiolipin availability may explain the protective effects of unsaturated fatty acids and we are currently examining this hypothesis.

Irrespective of the mechanisms involved, the current studies have revealed that mono-unsaturated fatty acids have the unique capacity to antagonise apoptosis induced by the activation of several different pathways in pancreatic β-cells. Since these pathways are regulated by agents implicated in the loss of pancreatic β-cells in both type 1 (cytokines) and type 2 (saturated fatty acids) diabetes, this suggests that mono-unsaturated fatty acids may have the potential to reduce the rate of β-cell loss associated with these conditions.

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