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RESEARCH PAPER

Pharmacological characterization of the cytoprotective effects of polyunsaturated fatty acids in insulin-secreting BRIN-BD11 cells

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BACKGROUND AND PURPOSE

Free fatty acids are important metabolic fuels for mammalian cells but, recently, it has become clear that they can also fulfil signalling functions, which are independent of their metabolic fate. We are investigating the ability of unsaturated free fatty acids to exert a cytoprotective response during exposure of insulin-secreting cells to toxic stimuli. The majority of earlier studies have focussed on monounsaturated fatty acids but this has now been extended to define the structural requirements of the cytoprotective effects of polyunsaturated species.

EXPERIMENTAL APPROACH

Clonal rat insulin-secreting cell lines, BRIN-BD11 or INS-1, were exposed to fatty acids or their derivatives complexed with BSA and the viability of the cells was analysed by flow cytometry after staining with propidium iodide.

KEY RESULTS

A variety of polyunsaturated fatty acids with chain lengths between C18–C22 attenuated the cytotoxic actions of the saturated fatty acid, palmitate (C16:0) in BRIN-BD11 and INS-1 cells. These effects were dose-dependent and displayed potencies that were much higher than those achieved with monounsaturated fatty acids. Methyl esters of the polyunsaturates were also effective. The cytoprotective responses were not altered by incubation of cells with inhibitors of cyclooxygenase or lipoxygenase enzymes although they were antagonized dose-dependently by arachidonyltrifluoromethylketone ($AACOCF_3$).

CONCLUSIONS AND IMPLICATIONS

The results are consistent with the involvement of a specific fatty acid binding site having loose, but defined, structural criteria, in mediating the cytoprotective effects of unsaturated fatty acids. $AACOCF_3$ may be of value in defining this site in molecular terms.

Abbreviations

AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; BEL, bromoenol lactone; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; FFA, free fatty acid; MAFP, methyl arachidonylfluorophosphonate; NDGA, nordihdroguaretic acid; $PACOCF_3$, palmitoyltrifluoromethylketone; PGE_2, prostaglandin E_2; PI, propidium iodide

Introduction

Free fatty acids (FFAs) are important metabolic fuels in mammalian cells but, recently, it has become apparent that they can also fulfil certain signalling functions, which are independent of their metabolic fate. Signalling activity occurs as a consequence of the interaction of FFAs with various receptor molecules expressed either intracellularly or on the plasma membrane of cells (Stoddart *et al.*, 2008; Ichimura *et al.*, 2009; Morgan and Dhayal, 2009; Obinata and Izumi, 2009; Wellendorph *et al.*, 2009; Sun *et al.*, 2010). Among these are transcription factors, such as the peroxisome proliferator activator receptors, which regulate gene expression, and a variety of cell surface molecules, which display the classical seven transmembrane domain organization characteristic of G-protein coupled receptors (Briscoe *et al.*, 2003; Stoddart *et al.*, 2008; Sugden and Holness, 2008; Swaminath, 2008; Morgan and Dhayal, 2009; Obinata and Izumi, 2009; Wellendorph *et al.*, 2009). As might be expected of receptor-mediated events, the signalling functions of FFAs display clear structure–activity relationships, which depend on factors such as chain length, degree of unsaturation, configuration of double bonds, etc. (Sum *et al.*, 2007; Dhayal *et al.*, 2008; Tikhonova *et al.*, 2008; Morgan and Dhayal, 2009). Nevertheless, it is also clear that considerable tolerance exists, with many FFA receptors having affinity for a variety of structurally diverse ligands (Briscoe *et al.*, 2003; Hirasawa *et al.*, 2008; Hara *et al.*, 2009).

We have been studying the ability of unsaturated FFAs to exert a cytoprotective response during exposure of pancreatic β-cells to toxic stimuli and are investigating whether this could derive from the activation of one or more FFA receptors (Diakogiannaki *et al.*, 2007; Dhayal *et al.*, 2008; Morgan and Dhayal, 2009). Evidence in support of this idea has accumulated as, for example, the cytoprotective response displays clear structure–activity relationships among fatty acids; it is of relatively high potency and is reproduced with essentially equal potency by both FFAs and certain of their poorly metabolized analogues (Dhayal *et al.*, 2008).

To date, the majority of studies have focussed on monounsaturated species and it has become clear that several monounsaturates, notably palmitoleate (C16:1) and oleate (C18:1), exert powerful cytoprotective effects in β-cells (Eitel *et al.*, 2002; Maedler *et al.*, 2003; Welters *et al.*, 2004; Diakogiannaki *et al.*, 2007). Indeed, these FFAs antagonize the toxicity of a range of different stimuli, including the 'lipotoxicity' associated with chronic exposure to saturated fatty acids as well as the loss of viability seen after treatment with pro-inflammatory cytokines or following the withdrawal of survival factors present in serum (Welters *et al.*, 2004; Dhayal *et al.*, 2008). As such, these observations are consistent with the possibility that unsaturated FFAs may promote cell viability by regulating a distal step in the pro-apoptotic pathway in insulin-secreting cells.

Although the majority of studies to date have employed monounsaturated fatty acids as the cytoprotective species, increasing evidence indicates that cytoprotection is not restricted solely to these molecules as polyunsaturated FFA molecules also inhibit the actions of cytotoxic stimuli in pancreatic β-cells (Beeharry *et al.*, 2003; Papadimitriou *et al.*, 2007; Wei *et al.*, 2009). However, the structure–activity relationships required for this response have not been examined in detail. Therefore, in the present work, we have characterized the functional pharmacology of a range of polyunsaturated FFAs in order to establish their structure–activity relationships as regulators of pancreatic β-cell viability, using BRIN-BD11 and INS-1 cells as models.

Methods

Cell culture

BRIN-BD11 and INS-1 cells were used in the present study. BRIN-BD11 and INS-1 cell lines were derived from a rat insulinoma by methods reported by McClenaghan *et al.* (McClenaghan *et al.*, 1996) and Asfari *et al.* (Asfari *et al.*, 1992) respectively. Both were cultured in RPMI-1640 medium containing 11 mM glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. The medium used to culture INS-1 cells was further supplemented with 50 μM β-mercaptoethanol. The cells were grown in monolayers at 37°C and in a humidified atmosphere containing 5% CO_2. For individual experiments, cells were seeded into six-well plates at a density of 1×10^5 cells per well for BRIN-BD11 cells and 1.5×10^5 cells per well for INS-1 cells in complete medium for 24 h. After this period, the medium was removed and replaced by serum-free medium containing appropriate fatty acid–BSA complexes.

Preparation of fatty acid–BSA complexes

Stock solutions of palmitate (50 mM) were prepared in 50% ethanol by heating to 70°C, and stock solutions of all monounsaturated and polyunsaturated fatty acids (at up to 90 mM) were prepared in 90% ethanol at room temperature. Fatty acids were bound to 10% fatty acid-free BSA by incubation at 37°C for 1 h in a solution containing 5% ethanol. The final concentration of BSA used for cell incubations was maintained at 1% and that of ethanol was maintained at 0.5%. Relevant vehicle (BSA plus ethanol) controls were included in each experiment and exerted no effect on cell viability.

Quantification of cell death by flow cytometry

Following incubation, both live and dead cells were collected, centrifuged at 300× *g* for 5 min and the pellet resuspended in 200 μL of medium. Propidium iodide (PI) staining solution was prepared by mixing 20 $\mu g \cdot mL^{-1}$ of PI with FACS buffer (PBS, 2% FCS, 10 mM sodium azide). PI solution (200 μL) was then added to the samples and incubated on ice for 10 min. The samples were then analysed on a Beckman-Coulter Epics XL.MCL flow cytometer running EXPO32 ADC software (Applied Cytometry Systems) software.

Prostaglandin E_2 measurement

BRIN-BD11 cells were grown in 24-well plates at a density of 7×10^4 cells per well in complete medium for 24 h. After this period, cells were treated with either 10 μM arachidonic acid (AA) or 10 μM AA methyl ester for 4 h in serum-free medium. At the end of the incubation period, the culture medium was

collected and prostaglandin E_2 (PGE_2) measured by ELISA according to the manufacturer's instructions.

Statistical analysis

All experiments were performed on at least three separate occasions using either duplicates or triplicates for each experimental condition. The results are expressed as mean values ± SEM and the level of significance was calculated using Student's *t*-test.

Materials

RPMI-1640 medium, penicillin/streptomycin and glutamine were purchased from Invitrogen (Paisley, Scotland). Fetal calf serum was purchased from PAA Laboratories (Somerset, England). Fatty acid-free BSA and palmitoleate were from MP Biomedicals (Oxon, England). γ-Linolenic acid, eicosapentanoic acid (EPA), docosahexanoic acid (DHA), adrenic acid, AA, 5,8,11,14-eicosatetraynoic acid (ETYA), bromoenol lactone (BEL), arachidonyltrifluoromethyl ketone ($AACOCF_3$), palmitoyltrifluoromethylketone ($PACOCF_3$) and methyl arachidonylfluorophosphonate (MAFP) were purchased from Enzo Life Sciences (Exeter, England). Indomethacin, nordihdroguaretic acid, palmitate, oleate, AA methyl ester, DHA methyl ester and PI were from Sigma (Dorset, England) and the PGE_2 ELISA kit was obtained from Thermo Fisher Scientific (Northumberland, England).

Results

Polyunsaturated fatty acids protect pancreatic β-cells against the 'lipotoxicity' caused by long chain saturated fatty acids

It is well established that exposure of cultured primary β-cells as well as clonal insulin-secreting cell lines to the long chain saturated fatty acid palmitate (C16:0) leads to loss of viability (Welters *et al.*, 2004; Diakogiannaki *et al.*, 2007; Jeffrey *et al.*, 2008; Chu *et al.*, 2010; Kim *et al.*, 2010). Accordingly, when rat BRIN-BD11 cells were exposed to 250 μM palmitate in tissue culture, ~80% of the cells were non-viable after 24 h as judged by vital dye staining or flow cytometric analysis (Figure 1). Co-incubation of the cells with 250 μM palmitate plus a monounsaturate, oleate (C18:1) or with either of two polyunsaturated C18:3 fatty acids, 9,12,15-octadecatrienoic acid (α-linolenic acid) or 6,9,12-octadecatrienoic acid (γ-linolenic acid) led to dose-dependent cytoprotection (Figure 1A).

In view of this result, we next examined the cytoprotective responses of polyunsaturated fatty acids having still longer chain lengths and more double bonds. This revealed that ω-3 species such as 5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5) and 4,7,10,13,16,19-docosahexaenoic acid (DHA; C22:6) were also powerfully cytoprotective against palmitate toxicity in BRIN-BD11 cells (Figure 1B). More strikingly, their apparent potencies (EC_{50}: EPA ~ 2.5 μM; DHA ~ 5 μM) were greater than that seen when the C18 species were used. Further studies revealed that this response was not restricted to ω-3 FFAs as polyunsaturated ω-6 molecules, adrenic acid (7,10,13,16-docosatetraenoic acid) and AA (5,8,11,14-eicosatetraenoic acid) also dose-dependently protected against palmitate toxicity (Figure 1C).

Similar experiments were repeated in a different rat insulin-secreting cell line, INS-1 (Figure 2) and in a relevant mouse cell line, MIN-6 (Figure S1) to ensure that the observed effects were not restricted to BRIN-BD11 cells.

Polyunsaturated fatty acids protect against the loss of viability caused by serum withdrawal

In addition to their ability to protect BRIN-BD11 cells against the cytotoxic actions of palmitate, a range of polyunsaturates also improved the viability of cells cultured in the absence of serum (Figure 3). Withdrawal of serum resulted in more than 70% loss of cell viability over a 30 h period and this was dose-dependently opposed by incubation with EPA, DHA, arachidonate or adrenic acid (Figure 3).

Inhibitors of cyclooxygenase and lipoxygenase enzymes fail to prevent the cytoprotective actions of polyunsaturated fatty acids

Polyunsaturated fatty acids can be metabolized in cells to yield a wide variety of biologically active eicosanoids, some of which are known to be functionally active in β-cells (Metz, 1988; Robertson, 1988; Persaud *et al.*, 2007; Keane and Newsholme, 2008). In particular, the cyclooxygenase and lipoxygenase pathways have been shown to be involved in the metabolism of AA in these cells (Metz, 1988; Turk *et al.*, 1993; Pek and Nathan, 1994) and the effects of inhibitors of these enzymes were therefore examined. Neither indomethacin (cyclooxygenase and lipoxygenase inhibitor), nordihdroguaretic acid (lipoxygenase inhibitor) nor the stable AA analogue, 5,8,11,14-eicosatetraynoic acid (ETYA; an inhibitor of both cyclooxygenases and lipoxygenases) caused any diminution in the cytoprotective effects of either AA (Figure 4A,B) or of EPA (Figure 4C). Indeed, the inhibitors did not modify the dose–response relationship for cytoprotection suggesting that the fatty acids were fully cytoprotective under conditions when cyclooxygenase and lipoxygenase activities were reduced. The functionality of relevant inhibitors was confirmed by measurement of PGE_2 production from BRIN-BD11 cells. This resulted in marked inhibition when compared with untreated cells [control: 2.0 ± 0.02 pg PGE_2 per 1000 cells; indomethacin: 0.09 ± 0.03 pg per 1000 cells ($P < 0.001$); ETYA: 0.04 ± 0.02 pg per 1000 cells ($P < 0.001$)].

Poorly metabolizable methyl esters of polyunsaturated fatty acids are cytoprotective

As the data obtained so far implied that FFA metabolism via the cyclooxygenase or lipoxygenase pathway(s) was not required for cytoprotection, the effects of polyunsaturated fatty acid methyl esters were also tested. These molecules are esterified by the addition of a methyl substituent at the terminal carboxyl group and, as a result, they are not available for thio-esterification to Coenzyme-A. Hence, they are not activated for subsequent oxidation. In addition, fatty acid methyl esters are poorly utilized as substrates by cyclooxygenase or lipoxygenases. In support of this, we found that while exposure of BRIN-BD11 cells to 10 μM of unesterified AA resulted in increased PGE_2 production [control: 2.0 ± 0.02 pg

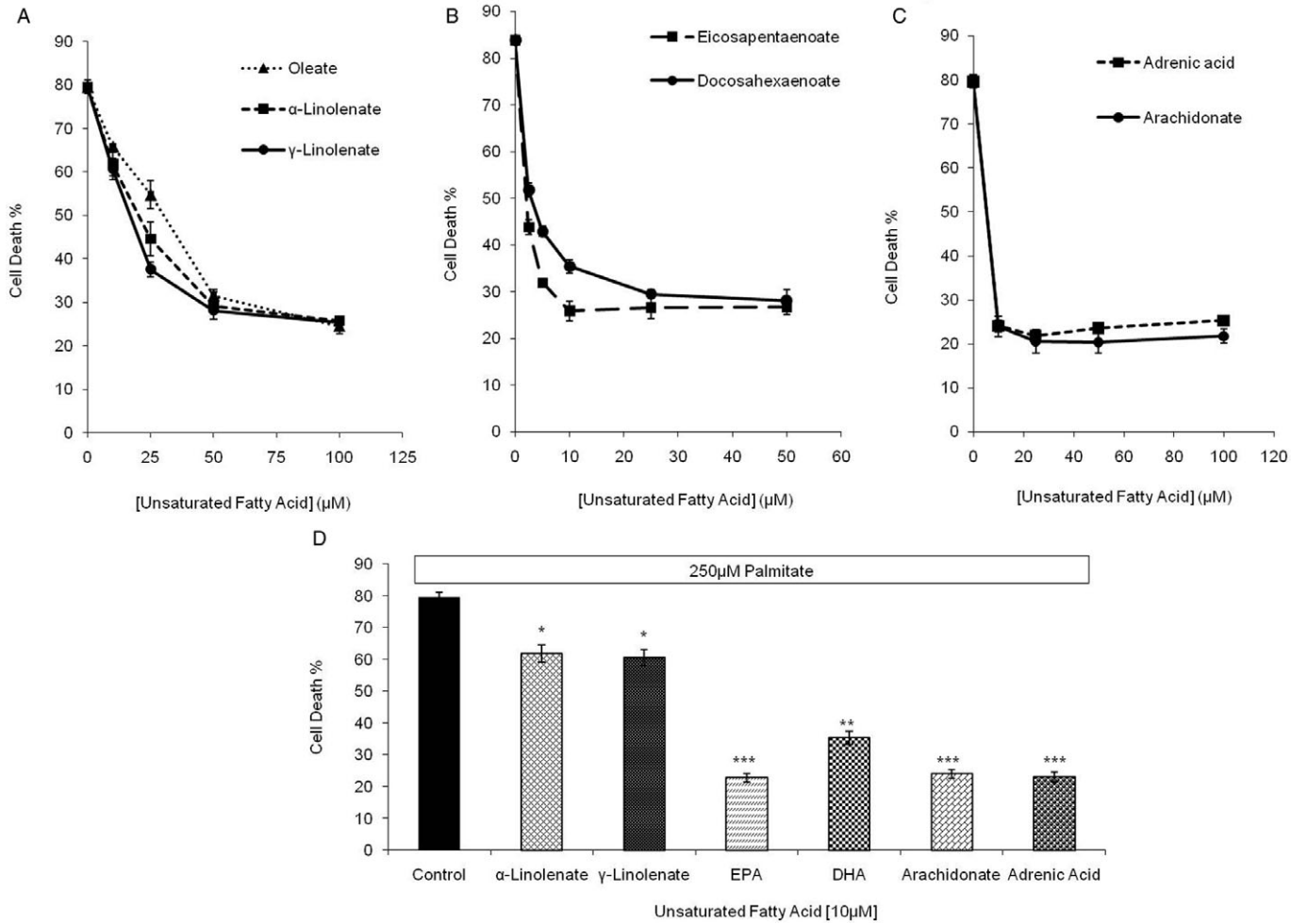

Figure 1

Effects of monounsaturated and polyunsaturated fatty acids on the toxic actions of palmitate in BRIN-BD11 cells. Cells were treated with 250 μM palmitate in the presence of increasing concentrations of (A) the monounsaturated fatty acid oleate or either of two polyunsaturated fatty acids, α-linolenate or γ-linolenate; (B) either eicosapentaenoate (EPA) or docosahexaenoate (DHA); (C) either adrenic acid or arachidonate; (D) cells were exposed to 250 μM palmitate in the presence of 10 μM of each of the polyunsaturated fatty acids shown and cell viability examined 18 h later. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ relative to palmitate alone.

per 1000 cells; AA: 3.9 ± 0.03 pg per 1000 cells ($P < 0.001$)] an equivalent concentration of AA methyl ester failed to increase PGE_2 formation (1.9 ± 0.02 pg per 1000 cells) under identical conditions. Despite this, AA methyl ester still supported the maintenance of cell viability during exposure to palmitate (Figure 5A). Similarly, the methyl ester of DHA also retained the cytoprotective activity of its parent fatty acid (Figure 5B).

The AA derivative, $AACOCF_3$, attenuates the cytoprotection mediated by unsaturated fatty acids

We next examined whether certain structurally modified polyunsaturated fatty acid analogues might influence cell viability. As outlined above, ETYA did not antagonize the cytoprotection afforded by AA in cells exposed to 250 μM palmitate (Figure 4A). However, the data shown in Figure 4A also reveal an additional feature; namely that, unlike AA, ETYA was not directly cytoprotective during exposure of cells to palmitate. Thus, despite bearing a close structural similarity to AA (Figure 6), the functional effects of these two molecules were quite different. This was also the case for a second AA analogue, MAFP (Figure 7), which neither exerted direct cytoprotection nor antagonized the protective response to AA. By contrast, a different effect was seen with a third derivative, arachidonyltrifluoromethylketone [1,1,1-trifluoro-6Z,9Z,12Z,15Z-heneicosateraen-2-one ($AACOCF_3$); Figure 8]. Like ETYA and MAFP, this agent did not, itself, reproduce the cytoprotection achieved with AA but, unlike ETYA and MAFP, it exerted an antagonistic effect when cells were incubated with AA in the presence of palmitate (Figure 8A). Thus, the presence of increasing concentrations of $AACOCF_3$ progressively reduced the extent of cytoprotection achieved with AA such that the lipotoxic actions of palmitate were again apparent. Even more strikingly, the antagonistic actions of $AACOCF_3$ were not confined to

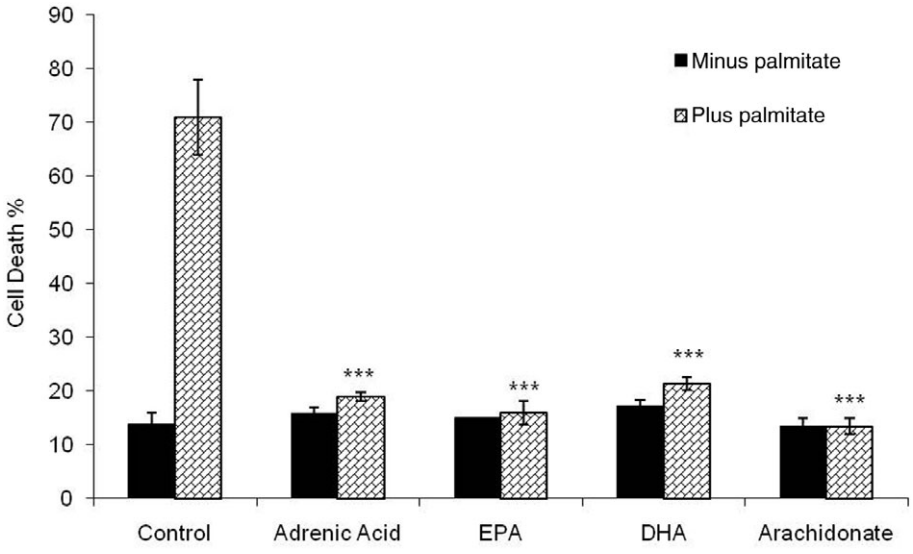

Figure 2

Effects of polyunsaturated fatty acids on the toxic actions of palmitate in INS-1 cells. INS-1 cells were exposed to 10 μM of various polyunsaturated fatty acids (EPA, DHA, arachidonate or adrenic acid, as indicated) in the absence or presence of 250 μM palmitate for 48 h. Cell viability was analysed at the end of this period of culture. ***$P > 0.001$, significantly different from palmitate alone.

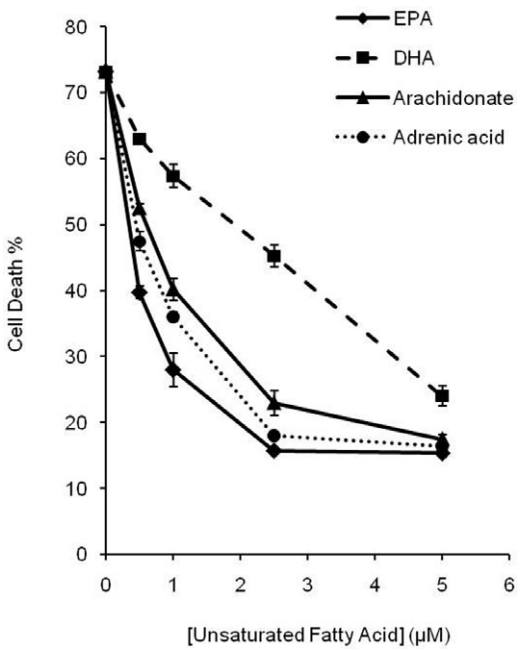

Figure 3

Effects of polyunsaturated fatty acids on apoptosis induced by withdrawal of serum from the culture medium. BRIN-BD11 cells were treated with increasing concentrations of various polyunsaturated fatty acids (EPA, DHA, arachidonate or adrenic acid, as shown) in serum-free medium for 30 h. Cell viability was analysed at the end of the culture period.

conditions under which AA was employed as the cytoprotective species as it also antagonized the protection afforded by EPA (Figure 8A) or by the monounsaturates oleate and palmitoleate (Figure 8B). $AACOCF_3$ also blocked the cytoprotection achieved with AA methyl ester in palmitate-treated cells (Figure S2).

To investigate this unexpected response further, the effect of $AACOCF_3$ was compared with that of two additional molecules, palmitoyltrifluoromethylketone [1,1,1-trifluoro-2-heptadecanone ($PACOCF_3$)] and bromoenol lactone [6E-(bromoethylene)tetrahydro-3S-(1-naphthalenyl)-2H-pyran-2-one; BEL]. $PACOCF_3$ is a palmitate derivative that contains the same trifluoro-substituent as $AACOCF_3$ in place of the terminal carboxyl group and BEL represents a structurally different molecule (Figure 6), which, in common with both $AACOCF_3$ and $PACOCF_3$, acts as an inhibitor of certain isoforms of phospholipase A_2 (Ma and Turk, 2001; Schaeffer *et al.*, 2005; Farooqui and Horrocks, 2006) (Figure 6). Neither BEL nor $PACOCF_3$ elicited any direct cytoprotective actions in cells exposed to 250 μM palmitate and, more importantly, these agents failed to reproduce the antagonism of AA-induced cytoprotection achieved with $AACOCF_3$ (Figure 9).

Discussion

The present work has revealed that the cytoprotection achieved by incubation of BRIN-BD11 and INS-1 cells with unsaturated FFAs is not limited to monounsaturated species but that a range of polyunsaturates are also effective. Indeed, the polyunsaturated fatty acids may be more potent than their monounsaturated counterparts in that the EC_{50} achieved with, for example, EPA or AA was some 5- to 10-fold lower than that obtained with palmitoleate (C16:1) or oleate (C18:1). Importantly, this means that a total added concentration of as little as 2.5 μM of EPA or AA abolished the cytotoxicity achieved by incubation of BRIN-BD11 cells with a much higher concentration (250 μM) of palmitate.

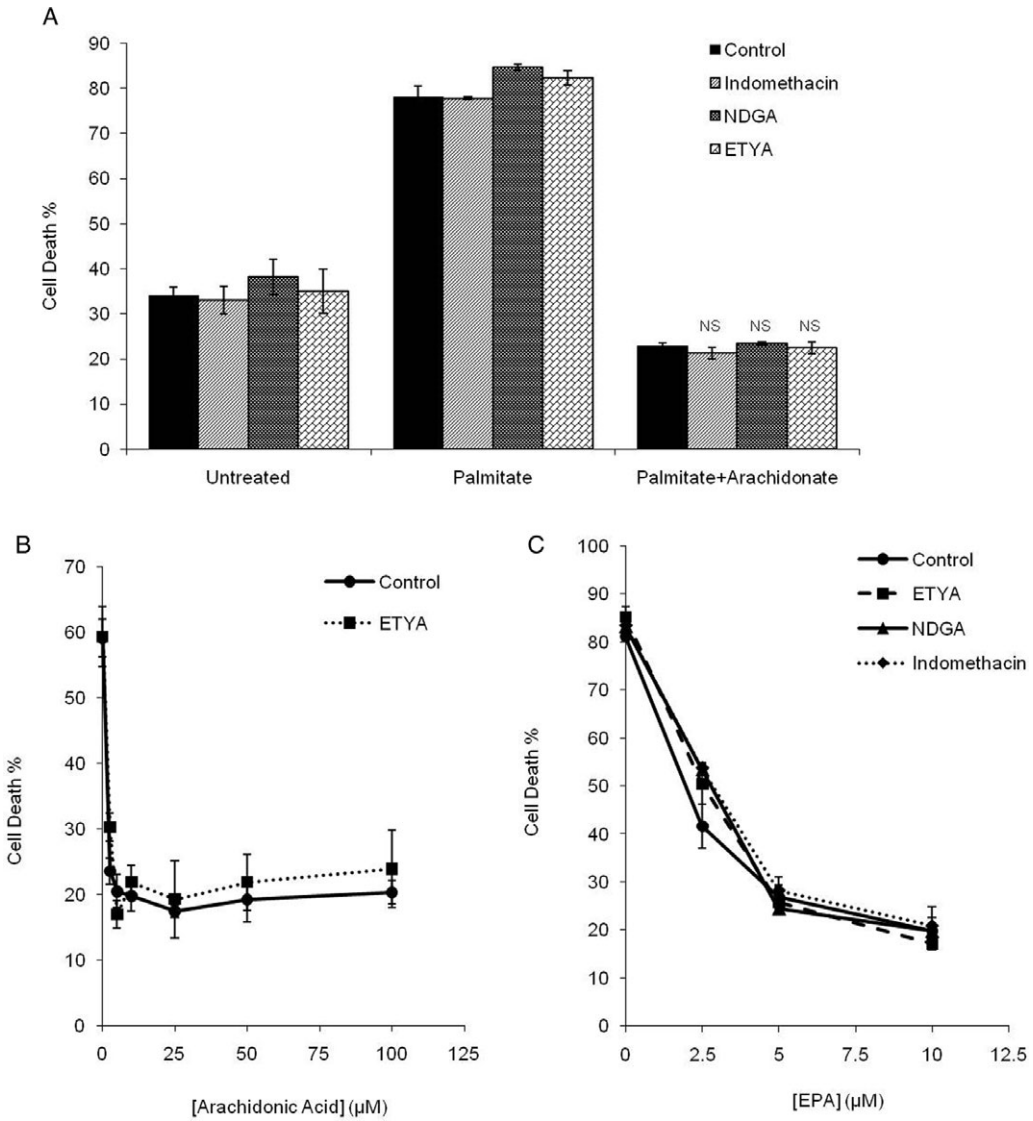

Figure 4

Effect of cyclooxygenase and lipoxygenase inhibitors on the cytoprotective effects of polyunsaturated fatty acids in cells exposed to palmitate. (A) BRIN-BD11 cells were incubated with vehicle (untreated), 250 μM palmitate or 250 μM palmitate plus 10 μM arachidonic acid in the absence or presence of 10 μM ETYA, 10 μM NDGA or 10 μM indomethacin, as shown. Cells were incubated for 18 h and viability was analysed thereafter. NS, not significantly different from palmitate plus arachidonate. (B) BRIN-BD11 cells were treated with 250 μM palmitate and increasing concentrations of arachidonate in the absence (control) or presence of 10 μM ETYA for 18 h. Cell viability was assessed at the end of this period. (C) BRIN-BD11 cells were treated with 250 μM palmitate in the presence of increasing concentrations of EPA in the absence (control) or presence of 10 μM ETYA, 10 μM NDGA or 10 μM indomethacin for 18 h. Cell viability was assessed at the end of this period.

In reflecting on the implications of this consideration, it is important to note that the effective free concentration of polyunsaturated fatty acids under our experimental conditions was likely to be much lower than the total added concentration quoted, as the fatty acids were delivered in complex with serum albumin. It is difficult to estimate the true free concentration of each fatty acid accurately due to uncertainties about the binding affinity of serum albumin for individual fatty acid species. Nevertheless, earlier calculations imply that the free concentration of polyunsaturated fatty acids is likely to lie in the subnanomolar range (Richieri *et al.*, 1993).

The apparent potency of the cytoprotective effects observed here is consistent with the possibility that they might be mediated by interaction of the polyunsaturated fatty acids with a critical binding site (possibly a fatty acid 'receptor') rather than via a metabolic action. Additional findings also support this conclusion. Thus, the methyl esters of polyunsaturated fatty acids were almost as potent as their unesterified parent fatty acids as cytoprotective agents. As fatty acyl methyl esters lack a free carboxyl group, they are not readily available for thio-esterification to Coenzyme-A. Hence, these molecules are poorly oxidized within cells and they are not readily incorporated into neutral lipids

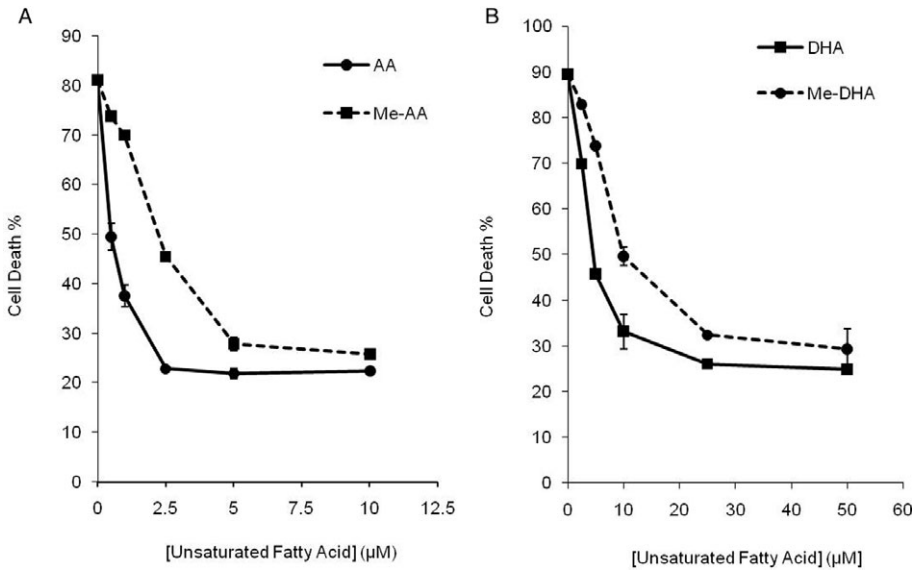

Figure 5

Effect of polyunsaturated fatty acid methyl esters on the toxic actions of palmitate in BRIN-BD11 cells. Cells were exposed to 250 μM palmitate in the presence of increasing concentrations of either arachidonate (AA) or arachidonate methyl ester (A) or either DHA or DHA methyl ester (B). Cells were incubated for 18 h and at the end of this period viability was analysed. Me, methyl ester.

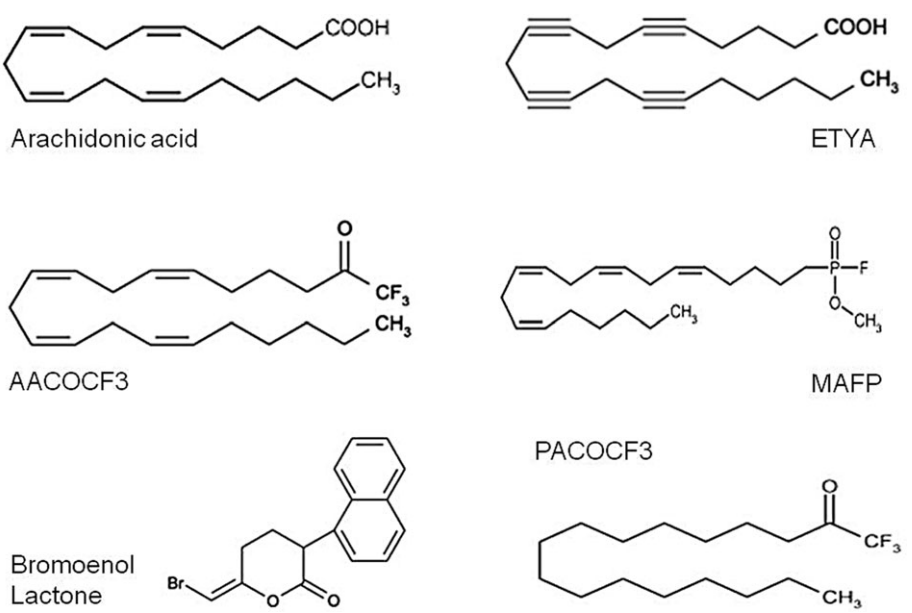

Figure 6

Structure of arachidonic acid and various phospholipase A_2 inhibitors used in the present study.

(Diakogiannaki *et al.*, 2008). Fatty acyl methyl esters are also not substrates for cyclooxygenase and lipoxygenase enzymes and we observed that incubation of BRIN-BD11 cells with AA methyl ester did not result in increased PGE_2 formation. By contrast, its parent FFA, AA, provoked a marked increase in the synthesis of this prostaglandin. This implies that the cytoprotective response to AA methyl ester did not result from rapid de-esterification to yield free AA. Hence, we conclude that both the poorly metabolized methyl esters of polyunsaturated fatty acids and the unesterified parent molecules are effective cytoprotective agents in insulin-secreting cell lines. As such, this suggests that the incorporation of exogenous fatty acids into neutral or phospholipids within the cell (which has been shown to occur in BRIN-BD11 cells (Diakogiannaki *et al.*, 2007) is unlikely to account for their cytoprotective actions.

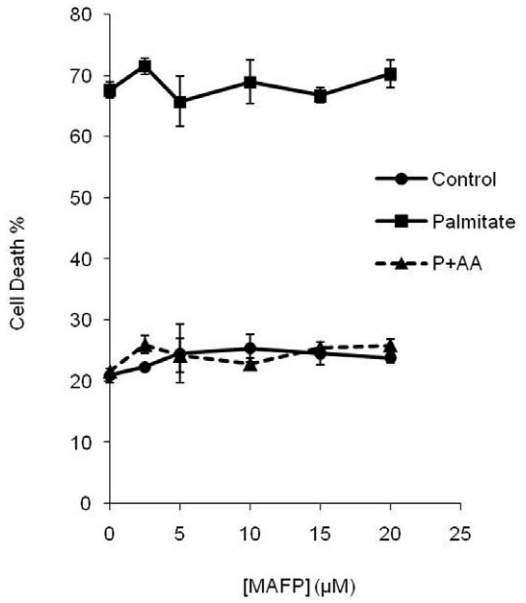

Figure 7

Effect of MAFP on the cytoprotection achieved with arachidonate in palmitate-treated BRIN-BD11 cells. Cells were incubated with vehicle (control), 250 μM palmitate alone or 250 μM palmitate plus 2.5 μM arachidonate (P + AA) in the presence of increasing concentrations of MAFP. Cell viability was assessed at the end of 18 h of incubation.

Recent studies have revealed that certain metabolites of polyunsaturated fatty acids, such as resolvins and (neuro)protectins, play important roles in cell signalling and that they can be cytoprotective (Mukherjee *et al.*, 2004; Serhan *et al.*, 2004; Das, 2008; Bazan, 2009; Spite *et al.*, 2009; Calandria and Bazan, 2010). However, the present data with the methyl esters of polyunsaturated fatty acids imply that generation of such molecules is unlikely to account for the cytoprotective actions of polyunsaturated fatty acids in BRIN-BD11 cells.

Taken together, these considerations point towards the conclusion that cytoprotection may derive from the interaction of relevant fatty acid ligands with a critical binding site, rather than from the generation of a specific intracellular metabolite. In view of this, we have aimed to establish, more fully, the pharmacological characteristics of the putative fatty acid binding site in functional terms.

One obvious feature that emerges from this analysis is that the putative 'receptor' must be capable of binding, in a productive manner, a range of fatty acids having rather different structural features. These include both monounsaturated fatty acids with a minimum chain length of C16, as well as various polyunsaturated fatty acids having a range of chain lengths and varying degrees of unsaturation. This is not necessarily inconsistent with receptor involvement as it is already clear that FFA receptors are tolerant to such variations (Briscoe *et al.*, 2003; Hirasawa *et al.*, 2008; Hara *et al.*, 2009). Nevertheless, the specificity of the cytoprotective response is not unrestricted as we have shown previously that it is sensitive to the configuration of the double bonds, with *cis*-unsaturates being more potent than their *trans* equivalents

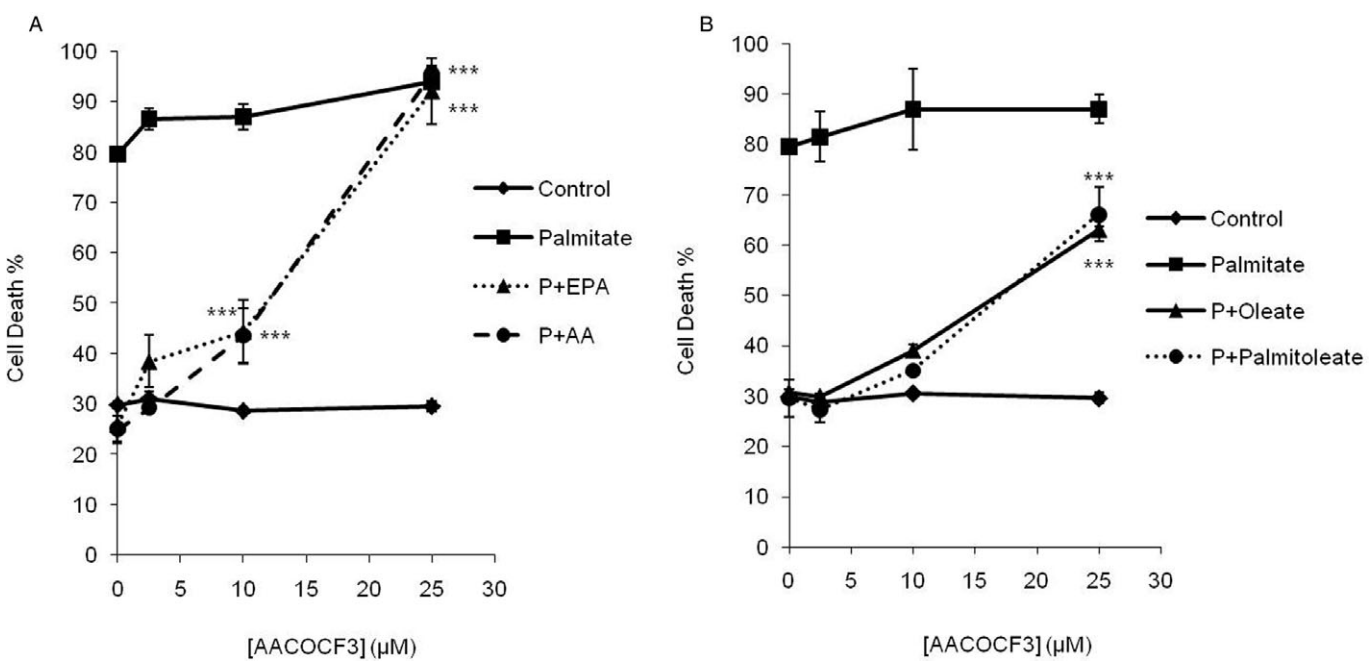

Figure 8

Effect of $AACOCF_3$ on the cytoprotection achieved with arachidonate in palmitate-treated BRIN-BD11 cells. Cells were treated with vehicle (control), 250 μM palmitate alone or 250 μM palmitate plus (A) 2.5 μM AA (P + AA) or 2.5 μM EPA (P + EPA) or (B) 50 μM palmitoleate (P + Palmitoleate) or 50 μM oleate (P + Oleate), in the presence of increasing concentrations of $AACOCF_3$. Cell viability was assessed at the end of 18 h of incubation. ***$P > 0.001$, significantly different from the equivalent incubation condition in the absence of $AACOCF_3$.

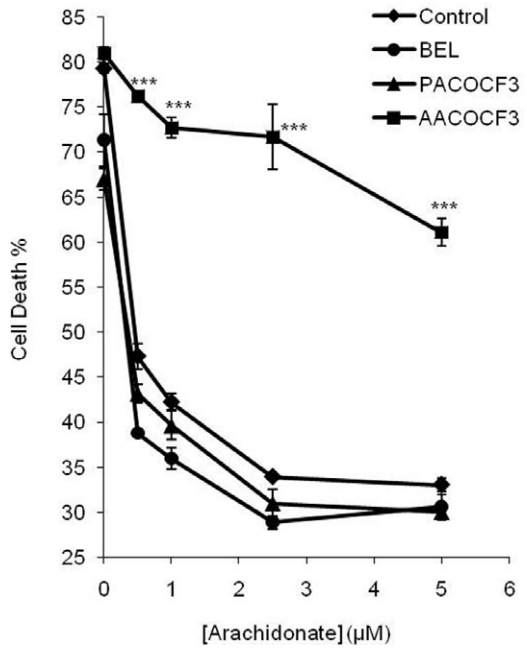

Figure 9

Effect of phospholipase A_2 inhibitors on the cytoprotection achieved with arachidonate in palmitate-treated BRIN-BD11 cells. Cells were treated with 250 μM palmitate in combination with increasing concentration of arachidonate in the presence of vehicle (control) or various phospholipase A_2 inhibitors: 10 μM BEL, 10 μM $PACOCF_3$ or 25 μM $AACOCF_3$ for 18 h. Cell death was analysed at the end of this incubation period. ***$P < 0.001$ significantly different from the equivalent concentration of the control series.

(Dhayal *et al.*, 2008). From the present work, it is noteworthy too, that ETYA did not reproduce the direct cytoprotective actions of AA when incubated with cells exposed to palmitate. ETYA is a close structural analogue of AA in which the principal difference is that the four double bonds present in the latter are replaced by triple bonds (Figure 8). Nevertheless, despite this apparently high degree of structural similarity, ETYA did not mediate a cytoprotective response in BRIN-BD11 cells exposed to 250 μM palmitate.

A further important finding is that a trifluorinated ketone derivative of AA, $AACOCF_3$, acted as an antagonist of the cytoprotection mediated by polyunsaturated fatty acids in BRIN-BD11 cells, while not causing any direct toxicity over the same concentration range. This was an unexpected result as, to the best of our knowledge, no other fatty acid or fatty acid derivative has been described, which possesses the capacity to act as an antagonist of the cytoprotective response mediated by an unsaturated fatty acid. Importantly, we found that $AACOCF_3$ antagonized the cytoprotection achieved not only by polyunsaturated fatty acids but also that caused by monounsaturated fatty acids. This should not be taken to imply that $AACOCF_3$ necessarily binds to the same site as the active fatty acids in order to mediate its antagonistic action, although this is an attractive possibility that is worthy of further consideration. In this context, it should also be noted that Zhang *et al.* (2008) have reported the suppression of EGF and bFGF-induced proliferation in human lens epithelial cells by $AACOCF_3$. These authors attributed this response to changes in redox signalling in the presence of $AACOCF_3$ (Zhang *et al.*, 2008). It is unclear whether a similar response occurs in BRIN-BD11 cells and, if so, how this might culminate in selective attenuation of the cytoprotective response mediated by other fatty acids. Interestingly, $PACOCF_3$, a palmitoyl trifluoroketo-derivative of the saturated fatty acid palmitate, was essentially inert in the cytoprotection assays conducted in our study. Presumably, the presence of a saturated species in this molecule prevents its interaction with the key site required for cytoprotection.

An important caveat to the considerations above is that $AACOCF_3$ has at least one additional, and well-described, activity; namely, that it acts as an inhibitor of some isoforms of phospholipase A_2 in cells (Street *et al.*, 1993; Loweth *et al.*, 1996). Thus, it is important to consider whether this activity could underlie its ability to attenuate the extent of cytoprotection seen when cells are exposed to polyunsaturated fatty acids or monounsaturated fatty acids in the presence of palmitate. In order to evaluate this possibility, we tested various other phospholipase A_2 inhibitors including BEL, MAFP and ETYA. As indicated above, ETYA and AA share a close structural similarity and this allows the former to act as an inhibitor of enzymes that utilize AA as substrate. Importantly, ETYA failed to antagonize the cytoprotective response to AA or other polyunsaturated fatty acids. Similarly, the data obtained with BEL, MAFP and $PACOCF_3$ also imply that inhibition of phospholipase A_2 isoforms is not the basis of cytoprotection.

Overall therefore, this study has revealed that certain polyunsaturated fatty acids exert potent cytoprotective actions in insulin-secreting cells. The response appears to be independent of their propensity to undergo β-oxidation and is not secondary to the generation of products arising from the metabolism of polyunsaturated fatty acids via the lipoxygenase or cyclooxygenase pathways. Rather, the data are consistent with the involvement of a specific binding site having loose, but defined, structural criteria. $AACOCF_3$ is shown to act as a functional antagonist of the cytoprotective response in BRIN-BD11 cells and may prove to be of value in defining this site in molecular terms, in future studies.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of arachidonic acid on the toxicity of palmitate in MIN-6 cells. MIN-6 cells were exposed to 10 μM of arachidonate in the absence or presence of 250 μM palmitate for 72 h. Cell viability was analysed at the end of this period of culture. ***$P > 0.001$ significantly different from palmitate alone.

Figure S2 Effect of $AACOCF_3$ on the cytoprotection achieved with methyl arachidonate in palmitate-treated BRIN-BD11 cells. Cells were treated with vehicle (control), 250 μM palmitate alone or 250 μM palmitate plus 5 μM arachidonate methyl ester (P + Me-AA) in the presence of increasing concentrations of $AACOCF_3$. Cell viability was assessed at the end of 18 h of incubation. ***$P > 0.001$ significantly different from palmitate plus arachidonate methyl ester in the absence of $AACOCF_3$.

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