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commentary

Arachidonic acid actions on functional integrity and attenuation of the negative effects of palmitic acid in a clonal pancreatic β-cell line

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ABSTRACT

Chronic exposure of pancreatic β-cells to saturated non-esterified fatty acids can lead to inhibition of insulin secretion and apoptosis. Several previous studies have demonstrated that saturated fatty acids such as PA (palmitic acid) are detrimental to β-cell function compared with unsaturated fatty acids. In the present study, we describe the effect of the polyunsaturated AA (arachidonic acid) on the function of the clonal pancreatic β-cell line BRIN-BD11 and demonstrate AA-dependent attenuation of PA effects. When added to β-cell incubations at 100 μM, AA can stimulate cell proliferation and chronic (24 h) basal insulin secretion. Microarray analysis and/or real-time PCR indicated significant AA-dependent up-regulation of genes involved in proliferation and fatty acid metabolism [e.g. *Angptl* (angiopoietin-like protein 4), *Ech1* (peroxisomal $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase), *Cox-1* (cyclo-oxygenase-1) and *Cox-2*, $P < 0.05$]. Experiments using specific COX and LOX (lipoxygenase) inhibitors demonstrated the importance of COX-1 activity for acute (20 min) stimulation of insulin secretion, suggesting that AA metabolites may be responsible for the insulinotropic effects. Moreover, concomitant incubation of AA with PA dose-dependently attenuated the detrimental effects of the saturated fatty acid, so reducing apoptosis and decreasing parameters of oxidative stress [ROS (reactive oxygen species) and NO levels] while improving the GSH/GSSG ratio. AA decreased the protein expression of iNOS (inducible NO synthase), the p65 subunit of NF-κB (nuclear factor κB) and the p47 subunit of NADPH oxidase in PA-treated cells. These findings indicate that AA has an important regulatory and protective β-cell action, which may be beneficial to function and survival in the 'lipotoxic' environment commonly associated with Type 2 diabetes mellitus.

Key words: arachidonic acid, lipotoxicity, palmitic acid, pancreatic β-cell, reactive oxygen species, Type 2 diabetes mellitus.

Abbreviations: AA, arachidonic acid; *Abcg1*, ATP-binding cassette subfamily G member 1; *Ampk*, AMP-activated protein kinase; COX, cyclo-oxygenase; DHE, dihydroethidine; *Ech1*, peroxisomal $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase; FBS, fetal bovine serum; *Gck*, glucokinase; GSIS, glucose-stimulated insulin secretion; HETE, hydroxyeicosatetraenoic acid; iNOS, inducible NO synthase; LT, leukotriene; NEFA, non-esterified fatty acid; NF-κB, nuclear factor κB; NS-398, *N*-(2-cyclohexyloxyl-4 nitrophenyl)methanesulfonamide; PA, palmitic acid; PI, propidium iodide; PG, prostaglandin; PLA_2, phospholipase A_2; *Ppara*, peroxisome-proliferator-activated receptor α; *Ppard*, peroxisome-proliferator-activated receptor δ; ROS, reactive oxygen species; RT–PCR (reverse transcription–PCR; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole; *Scd-2*, stearoyl-CoA desaturase-2; *Srebp-1c*, sterol-regulatory-element-binding protein-1c; T2DM, Type 2 diabetes mellitus; TNB, 2-nitro-5-thiobenzoic acid; UCP, uncoupling protein; WST-1, water-soluble tetrazolium salt 1; WT, wild-type.

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INTRODUCTION

T2DM (Type 2 diabetes mellitus) is a metabolic disorder characterized by elevated blood glucose concentrations. Most cases of T2DM are also characterized by elevated circulating fatty acid concentrations due to high dietary lipid intake, which is associated with decreased GSIS (glucose-stimulated insulin secretion) [1,2]. The impairment of insulin secretion has been attributed to insults and desensitization associated with chronic exposure of pancreatic β-cells to elevated glucose and NEFA (non-esterified fatty acid) levels, which can lead to reduction in β-cell mass (glucolipotoxicity) [3,4]. It is possible that NEFAs inhibit insulin secretion by slowing glucose uptake, metabolism and oxidation via Randle cycle effects, where an increase in availability of NEFA favours their oxidation leading to impaired glucose metabolism via glycolytic inhibition [5,6]. The exact mechanism of the cytotoxic effect of NEFA remains unclear, but it has been suggested that formation of free radicals [7,8], generation of ceramides [9], activation of endoplasmic reticulum stress and of stress-related response genes, such as that encoding NF-κB (nuclear factor κB) [10], could be involved in triggering apoptosis. *In vitro* studies have demonstrated that saturated fatty acids such as PA (palmitic acid) and stearic acid are more toxic than unsaturated fatty acid such as oleic and AA (arachidonic acid), although unsaturated fatty acids are not entirely free of cytotoxic effects at elevated concentrations [11–14]. NEFAs, however, in low concentrations are essential for GSIS by potentiation of GSIS and can be used as an energy substrate for β-cells during periods of fasting and starvation.

PA is one of the most abundant saturated fatty acids in the human diet and is the major fatty acid synthesized *de novo* in the liver; in addition, its levels are elevated in the plasma in T2DM [15,16]. Several studies have demonstrated the detrimental effect of chronic exposure (usually 24 h) of different pancreatic β-cell lines and rodent islets, to PA [17]. By contrast, AA is suggested to be an important modulator of pancreatic β-cell function, enhancing insulin secretion and cell proliferation *in vitro* [18]. The metabolism of AA by various isoforms of COX (cyclo-oxygenase) generates lipid products that can increase insulin secretion [16]. A recent study showed that concomitant incubation of BRIN-BD11 β-cells with inhibitors of AA mobilization altered glucose-induced insulin secretion when compared with cells incubated in the presence of AA [19]. BRIN-BD11 β-cells represent a useful model for such studies, since they are stable in culture and have well-characterized metabolic, signalling, insulin secretory and cell viability responses to glucose, amino acids and numerous other modulators of β-cell function (see [20,21] for details). Additionally, recently published work has reported that palmitic acid and cytokines induce effects on insulin secretion and p47^{phox} expression to a similar extent in both BRIN-BD11 cells and mouse islets [22].

We have now extended these studies to investigate the roles of AA in the regulation of β-cell functional integrity, insulin secretion, gene expression, ROS (reactive oxygen species) production and protection from the detrimental effects of PA.

MATERIALS AND METHODS

Reagents

RPMI 1640 medium, penicillin/streptomycin, FBS (fetal bovine serum) and glutamine were obtained from Gibco. The WST-1 (water-soluble tetrazolium salt 1) cell viability assay was obtained from Roche Diagnostics. The rat insulin ELISA kit was obtained from Mercodia. The Griess Reagent System for nitrite detection was obtained from Promega. All other reagents were obtained from Sigma–Aldrich unless otherwise stated.

Cell culture

BRIN-BD11 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 0.1% antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin) and 2 mM glutamine and were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air using a Forma Scientific incubator. Cells were kept between 1×10^5 and 1×10^6 cells/ml. For the experiments, cells (1.5×10^5) were seeded in a 24-well plate or containing 2 ml of medium or 1.5×10^6 in six-well plates containing 5 ml of medium and allowed to adhere overnight before treatment in the presence or absence of fatty acids. A stock solution of each fatty acid (100 mM) was prepared using ethanol as solvent. The final concentration of ethanol added to the cell culture medium was always less than 0.5%, a concentration that was not toxic to the cells (results not shown). In some experiments, PA and AA were prepared by mixing with 90% ethanol at room temperature (20°C) to produce stock solutions of 90 mM. The fatty acid preparations were then bound to 10% fatty-acid-free BSA (MP Biomedicals) by incubation for 1 h at 37°C. The mixture was added to RPMI 1640 medium (containing 11 mM glucose) deprived of FBS. The final concentrations present in the cell environment were 1% for BSA and 0.5% for ethanol. The cells were seeded into six-well plates at densities of 10^5 cells/well and incubated for 24 h in complete RPMI 1640 medium. The medium was then removed and replaced with relevant fatty acid–BSA complexes (in RPMI 1640 devoid of FBS) for a further 24 h. Controls received BSA and vehicle only.

Cell viability and relative cell number

Cell viability (or relative cell number) was assessed using an assay based on the reduction of WST-1,

a tetrazolium salt, to formazan by cell-dependent mitochondrial dehydrogenase activity. An increase in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. Briefly, BRIN-BD11 cells (2×10^4) were plated in a 96-well plate and allowed adhere overnight. Cells were treated for 24 h, and at the end of incubation, the cell proliferation assaying reagent WST-1 (10 μl) was added to each well. The cells were incubated for 1 h in the incubator, and the formazan dye produced was quantified by measuring its absorbance at 450 nm using a kinetic plate reader (Spectramax Plus; Molecular Devices). For cell viability studies using flow cytometry, fatty acids were bound to 10 % fatty-acid-free BSA by incubation at 37 °C for 1 h, and the final concentration of BSA was maintained at 1 %. For individual experiments, the culture medium was removed and replaced by serum-free RPMI 1640 containing appropriate fatty acid–BSA complexes. Controls received BSA and vehicle only.

Flow cytometry

Following incubation of cells with relevant fatty acids, both attached and floating cells were harvested and centrifuged at 300 *g* for 5 min. The pellet was resuspended in 200 μl of medium. PI (propidium iodide) staining solution was prepared by mixing 20 μg/ml PI with FACS buffer (PBS, 2 % FBS and 10 mM sodium azide). A portion (200 μl) of this PI solution 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 and analysed using EXPO32 ADC software (Applied Cytometry Systems).

Insulin secretion

After cell incubation, in the absence or presence of NEFA for 24 h, the medium was removed from each well and, in some cases, stored for later insulin measurement. The cells were then washed with PBS, and acute insulin secretion was determined after cell incubation for 40 min in KRB (Krebs Ringer buffer), pH 7.4, containing 1.1 mM D-glucose, followed by subsequent stimulation for 20 min in KRB pH 7.4 containing 16.7 mM glucose plus 10 mM alanine (a robust and reproducible insulinotropic stimulus) unless otherwise stated. The medium was then collected, and insulin secretion was determined using a Mercodia Ultra-sensitive Rat Insulin ELISA kit.

RT–PCR (reverse transcription–PCR)

Total RNA was isolated from cells using TRI REAGENT (Molecular Research Center) according to standard protocols provided by the manufacturer. cDNA synthesis was carried out on the total RNA using the Superscript Preamplification System II (Invitrogen Life Technologies). The primers used were *Srebp-1c* (sterol-regulatory-element-binding protein-1c) (forward primer, 5′-TCAGTTCCAGCATGGCTACC-3′; reverse primer, 5′-GCAGTTGATGTAGAGGCTAA-3′), *Ppara* (peroxisome-proliferator-activated receptor-α) (forward primer, 5′-GGTCCGATTCTTCCACTGC-3′; reverse primer, 5′-TCCCCTCCTGCAACTTCTC-3′), *Ppard* (peroxisome-proliferator-activated receptor-δ) (forward primer, 5′-GCAGGCTCTAGAATTCCATC-3′; reverse primer, 5′-GCCGTGTCTGGAGTGTTGTG-3′), *Ampk* (AMP-activated protein kinase) (forward primer, 5′-TGCTTTGCTGTGTGGAACTC-3′; reverse primer, 5′-TGTTGTACAGGCAGCTGAGG-3′), *Gck* (glucokinase) (forward primer, 5′-CAGTGGAGCGTGAAGACAAA-3′; reverse primer, 5′-ACCATCCGGTCATACTCCAG-3′), and *Ucp-2* (uncoupling protein-2) (forward primer, 5′-ATTGCACGAGAGGAAGGGAT-3′; reverse primer, 5′-ATTACTACGTTCAGGATCC-3′). Expression of genes of interest were normalized to *18S* rRNA amplified from the same samples. *18S* rRNA expression levels were not altered by any treatment reported in the present study.

Microarray

Total RNA from the cells was isolated using RNeasy kit (Qiagen). RNA samples were prepared for microarray analysis using the GeneChip System (Affymetrix). Briefly, cDNA synthesis was performed using the First-Strand cDNA Synthesis Kit. The double-stranded cDNA was then cleaned using the GeneChip Sample Cleanup module. Synthesis of biotin-labelled cRNA was then prepared using the GeneChip IVT Labelling kit. Subsequently, the biotin-labelled cRNA was cleaned using the GeneChip Sample Cleanup module and following quantification was fragmented at 94 °C prior to hybridization with Rat Genome 230 2.0 Array. After hybridization, the arrays were washed and stained on a GeneChip Fluidics Station (Affymetrix). The preliminary preprocessing and the comparative gene expression analysis of this data was carried out using freeware from the Bioconductor website (www.bioconductor.org). Transcripts representing genes with a value of >1.7-fold change between control and AA-treated samples and a P value < 0.05 were deemed to be significantly differentially expressed.

Real-time PCR

Total RNA was isolated from cells using TRI REAGENT (Molecular Research Center) according to the manufacturer's protocol. cDNA synthesis was carried out on the total RNA using the Superscript Preamplification System II (Invitrogen Life Technologies). Real-time PCR TaqMan assay was used to quantify the relative expression levels of genes identified as differentially expressed by microarray data. To eliminate the possibility of genomic DNA contamination, RNA samples (1 μg) were digested with DNase I before reverse transcription. DNase-treated RNA samples were then used as templates for

first-strand cDNA synthesis, using SuperScript II reverse transcriptase and random primers; 2 μl of cDNA was used for real-time PCR analysis. The probes for the target genes were labelled with the fluorescent dye, FAM on the 5′-end and a non-fluorescent quencher on the 3′-end. *18S* rRNA was used as an endogenous control for normalization of the target genes. PCR reactions were set up with Taqman Universal PCR Master Mix from Applied Biosystems. cDNA was amplified on the 7900HT Sequence Detection System (Applied Biosystems). Results were analysed using the relative standard curve method of analysis/ΔC_t (threshold cycle) method of analysis.

ROS detection

After incubation, cells were washed with PBS and lysed with lysis buffer (containing 1 % Triton-X100 and 40 mM EDTA). Lysates were then transferred to a 96-well opaque plate and incubated for 30 min with 10 μM H_2DCFDA (2′,7′,di-hydrochlorofluorescein-diacetate) (Molecular Probes). Its oxidation by ROS releases DCF (2′,7′-dichlorofluorescein), which is fluorescent (excitation/emission at 495/527 nm). Fluorescence was measured in a Spectramax Plus Fluorescence Plate Reader (Molecular Devices).

Superoxide production

Cells were seeded in an opaque 96-well plate, and after treatment, 10 μM DHE (dihydroethidine) was added and incubated for 30 min in the dark and under agitation. This probe is oxidized in the presence of superoxide, and ethidium is released. Ethidium is a fluorescent compound, which binds to the DNA. Its fluorescence was measured using a Spectramax Plus Fluorescence Plate Reader (Molecular Devices).

Nitrite production

NO production was measured indirectly by nitrite quantification, which is a stable NO oxidation product. Nitrite was measured using the Griess Reagent System (Promega) by following the manufacturer's protocol.

Intracellular GSH (reduced glutathione) and GSSG (oxidized glutathione) measurement

Intracellular GSH and GSSG content was determined using a protocol described by Rahman et al. [23]. The assay is based on the reaction of GSH with DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] (also known as Ellman's reagent), which produces the TNB (2-nitro-5-thiobenzoic acid) chromophore, which has a maximal absorbance at 412 nm, and GSSG–TNB adduct). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample. The disulfide product (GSSG–TNB) is then reduced by glutathione reductase in the presence of NADPH, recycling GSH back into the reaction.

Briefly, cells were lysed in an extraction buffer (0.1 % Triton X-100 and 0.6 % sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5) for 15 min under agitation and centrifuged at 12 000 ***g*** for 10 min. The supernatant was collected and stored at −80 °C. An aliquot was kept for protein measurement.

For GSH measurement, samples and GSH standards were added to a 96-well plate with a reaction mixture containing DTNB, β-NADPH and glutathione reductase, and the absorbance was read at 412 nm using a Spectramax Plus Plate Reader (Molecular Devices). Results are expressed as μg/mg of protein.

For GSSG measurement, samples and GSSG standards were incubated with 2-vinylpyridine and triethanolamine for 1 h at room temperature and then transferred to a 96-well plate and read at 412 nm. Results are expressed as μg/mg of protein.

Western blot analysis

After the incubation period, cells were trypsinized and centrifuged at 1200 ***g*** for 10 min and homogenized in extraction buffer (100 mM Tris/HCl, pH 7.0, 10 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate and 10 mM sodium orthovanadate). The samples were sonicated and SDS (10 %) was added, followed by 30 min of incubation on ice. After that, samples were centrifuged at 12 000 ***g***, for 40 min, at 4 °C. Aliquots of supernatants were used for measurement of total protein content as previously described [18]. Equal amounts of protein from each sample were separated using SDS/PAGE. Western blotting was carried out using standard protocols.

Statistical analysis

The results are presented as means ± S.E.M. and were analysed by one-way or two-way ANOVA with Tukey or Bonferroni post-hoc tests respectively. The level of significance was set at $P < 0.05$.

RESULTS

Effect of AA on relative cell number

Incubation of BRIN-BD11 cells with AA (up to 150 μM) for 24 h did not impair cell viability when compared with control, as determined by WST-1 analysis or flow cytometry. WST-1 is widely used to determine mitochondrial dehydrogenase activity, making the assumption that viable cells contain fully functional mitochondria, and therefore WST-1 absorbance is proportional to cell number. The addition of 100 μM of AA, however, stimulated cell proliferation, increasing the cell population by 19 % when compared with the control. Incubation of the BRIN-BD11 cells with a relatively high

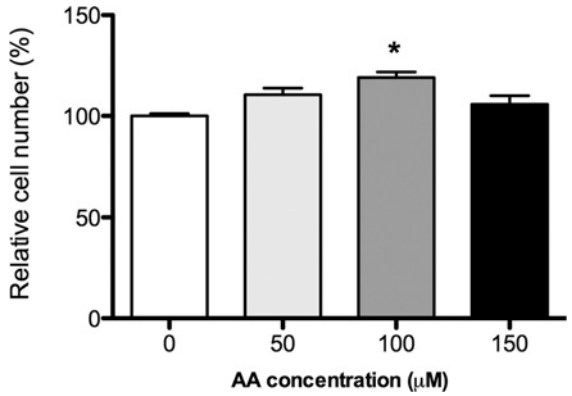

Figure 1 Effect of AA on relative cell number

BRIN-BD11 β-cells were incubated in the absence or presence of various concentrations of AA for 24 h (50–150 μM AA). Relative cell number was assessed using an assay based on the reduction of WST-1, a tetrazolium salt, to formazan by cellular mitochondrial dehydrogenase activity. An increase in the number of viable cells results in an increase in the overall mitochondrial dehydrogenase activity. Results are normalized to 100% and are expressed as means ± S.E.M., for six independent experiments. $^*P < 0.05$ compared with WST-1 absorbance from control cells.

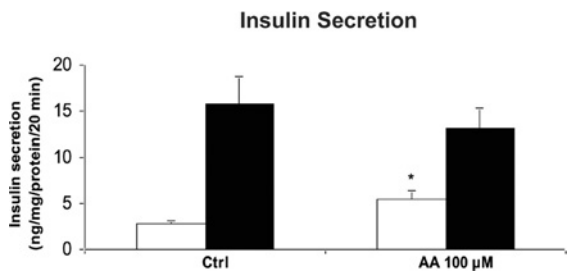

Figure 2 Effect of AA on acute (20 min) insulin secretion

BRIN-BD11 β-cells were incubated in the absence (Ctrl) or presence of 100 μM AA for 24 h. Subsequently, an acute (20 min) determination of basal- (1.1 mM glucose; black bars) and nutrient- (16.7 mM glucose and 10 mM alanine; white bars) stimulated insulin secretion was made. Results are expressed as means ± S.E.M., for six independent experiments. $^*P < 0.05$ compared with basal insulin secretion from control cells.

level of 150 μM AA resulted in a decrease in cell viability and therefore a reduction in relative cell number when compared with cells incubated with 100 μM, but not when compared with the vehicle control (Figure 1).

Effect of AA on acute (20 min) insulin secretion

A significant increase in basal- (1.1 mM glucose), but not nutrient-, (16.7 mM glucose and 10 mM alanine) stimulated (20 min) insulin secretion ($P < 0.05$) was observed following 24 h exposure to 100 μM AA, when compared with the vehicle control (Figure 2).

Effect of AA on gene expression

RT–PCR analysis comparing *Srebp-1c*, *Ppard*, *Ppara*, *Ins* (proinsulin), *Ucp-2*, *Ampk* and *Gck* mRNA expression in BRIN-BD11 cells treated in the absence or presence of increasing concentrations of AA for 24 h was performed. A significant ($P < 0.05$) increase in the relative expression of *Ampk* mRNA was observed in BRIN-BD11 cells stimulated for 24 h with 100 μM AA, compared with untreated control cells.

There was a significant ($P < 0.01$) decrease in relative expression of *Srebp-1c* following 24-h exposure to 50 or 100 μM AA compared with cells cultured in control medium, whereas the relative expression levels of *Ppara* and *Ppard* mRNA in cells treated with 50 or 100 μM AA was also significantly ($P < 0.05$) reduced compared with control cells. However, there was no significant change in proinsulin, *Ucp-2* or *Gck* mRNA expression following 24-h stimulation with AA.

Microarray analysis and subsequent data analysis (using freeware from the Bioconductor website (www.bioconductor.org) revealed that four genes were significantly altered in response to chronic (24 h) treatment of BRIN-BD11 cells with 100 μM AA (Table 1). The down-regulation of *Scd-2* (stearoyl-CoA desaturase) and *Abcg1* (ATP-binding cassette subfamily G member 1) observed using microarray analysis was confirmed using real-time PCR (Figures 3A and 3B). The up-regulation of *Ech1* (peroxisomal $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase) and *Angptl4* (angiopoietin-like protein 4), both detected by microarray analysis, was also confirmed by using real-time PCR (Figures 3C and 3D).

Effect of COX/LOX (lipoxygenase) inhibitors in combination with AA on insulin secretion

In order to determine whether the effects of AA on β-cell function were mediated by the products of AA metabolism or by AA itself, specific COX enzyme inhibitors and a non-selective LOX enzyme inhibitor were added to the cell culture medium for 24 h prior to the determination of acute nutrient stimulated insulin secretion (Figure 4). The selectivity of various COX inhibitors has recently been reviewed [24].

Selective inhibition of COX-1 by 100 μM acetaminophen decreased significantly ($P < 0.05$) the extent of nutrient-stimulated insulin secretion in the presence of 100 μM AA. This finding suggests that the inhibition of the COX-1 pathway results in a loss of AA-derived metabolites, which contribute to D-glucose- and L-alanine-stimulated insulin secretion. No differences were found in basal insulin secretion. Inhibition of COX-1 with 10 μM SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole] in combination with 100 μM AA, however, significantly ($P < 0.05$) enhanced insulin secretion measured under non-stimulating

Table 1 Microarray gene expression analysis following 24 h of AA treatment

List of BRIN-BD11 β-cell genes differentially regulated >1.7-fold following 24-h incubation with 100 μM AA compared with vehicle control.

Function	GenBank® accession no.	Gene name	Fold change	*P* value
Lipid metabolism	NM_031841	Stearoyl-CoA desaturase-2 (*Scd-2*)	−3.87	0.0018
Cholesterol transport/lipid trafficking	NM_053502	ATP-binding cassette transporter subfamily G member 1 (*Abcg1*)	−1.7	0.0284
Angiogenesis/differentiation	AA818262	Angiopoietin-like protein 4 (*Angptl4*)	+2.72	0.0134
Fatty acid metabolism mitochondrial and peroxisomal	NM_022594	Peroxisomal $\Delta^{3,5},\Delta^{2,4}$-dienoyl CoA isomerase (*Ech1*)	+1.73	0.0284

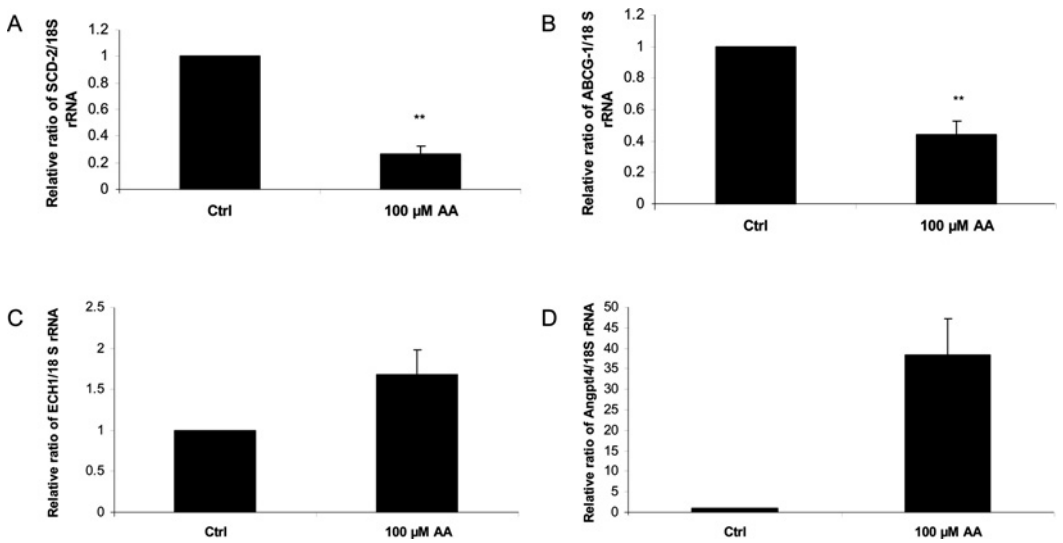

Figure 3 Effect of AA on gene expression as determined by real-time PCR.

BRIN-BD11 β-cells were cultured in the absence (Ctrl) or presence of 100 μM AA for 24 h. Expression of (A) *Scd-2*, (B) *Abcg-1*, (C) *Ech1* and (D) *Angptl4* was determined by real-time PCR and normalized to *18S* rRNA amplified from the same samples. Results are means $\pm$ S.E.M. for three individual experiments where, for each condition, they were performed in duplicate. The relative ratio for the control cells was arbitrarily set to 1. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control cells.

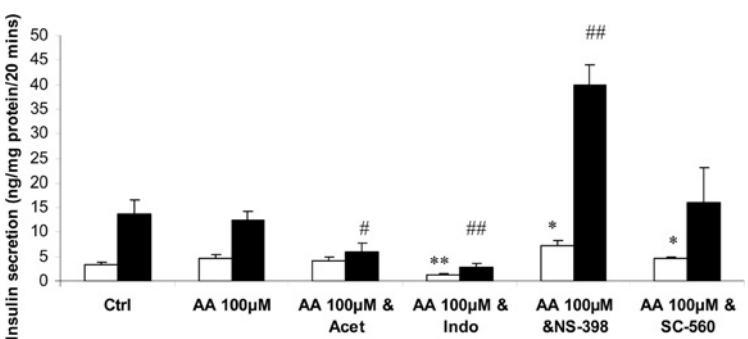

Figure 4 Effect of COX/LOX inhibitors in combination with AA on insulin secretion

BRIN-BD11 β-cells were incubated in the absence (Ctrl) or presence of 100 μM AA or 100 μM AA in combination with 100 μM acetaminophen (Acet) or 100 μM indomethacin (Indo) or 100 μM NS-398 or 10 μM SC-560 for 24 h. Subsequently, an acute (20 min) determination of basal- and nutrient- (D-glucose and L-alanine) stimulated insulin secretion was obtained. Results are expressed as means $\pm$ S.E.M. for six independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with cells incubated with control medium; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with cells incubated with 100 μM AA and stimulated with 16.7 mM D-glucose and 10 mM L-alanine.

conditions compared with control. There was no significant effect on nutrient-stimulated insulin secretion from cells incubated with SC-560 and AA.

Combinations of 100 μM indomethacin (a non-specific COX and LOX inhibitor) with 100 μM AA significantly reduced basal- ($P < 0.01$) and nutrient- ($P < 0.01$) stimulated insulin secretion when compared with control.

Selective inhibition of COX-2 via 100 μM NS-398 [*N*-(2-cyclohexyloxyl-4 nitrophenyl)methanesulfonamide] in the presence of 100 μM AA significantly enhanced basal- ($P < 0.05$) and nutrient- ($P < 0.01$) stimulated insulin secretion when compared with control. The increase in nutrient-stimulated insulin secretion in the presence of NS-398 and exogenous AA was also significantly elevated ($P < 0.01$) when compared with BRIN-BD11 cells incubated with 100 μM AA alone.

Effect of PA or AA on expression levels of COX-1 and COX-2

The incubation of BRIN-BD11 cells with PA for 24 h did not change the expression levels of *Cox-1* or *Cox-2* mRNA (Figure 5). Incubation with AA (100 μM) alone or in combination with PA, significantly increased the expression of both enzymes when compared with the no-AA control. Additionally, concomitant incubation of PA and AA (100 μM) for 24 h significantly reduced the expression levels of *Cox-1* and *Cox-2* mRNA, compared with AA alone, in a PA-concentration-dependent manner.

Rescue of PA-incubated cells by AA

Incubation of BRIN-BD11 cells with PA for 24 h decreased relative cell number in a dose-dependent manner as determined by WST-1 analysis ($P < 0.001$; Table 2). However, concomitant incubation with AA protected BRIN-BD11 cells from the toxic effect of PA in a dose-dependent manner as determined either by WST-1 analysis (Table 2) or by flow cytometry (Figure 6A). The two experiments described in Table 2 and Figure 6(A) were performed using different methods of fatty acid delivery. The fatty acids were added to the incubation medium in ethanol only in the experiment described in Table 2, while the fatty acids were delivered complexed to BSA in the experiment described in Figure 6(A). The latter revealed that the cytoprotective response was extremely potent with an EC_{50} $\sim$2 μM (total AA concentration) under the experimental conditions used and that AA caused complete protection against the cytotoxicity induced by 250 μM PA.

Effect of PA and AA on insulin secretion measured over 24 h

PA decreased cumulative insulin secretion over a 24-h incubation period in a dose-dependent manner ($P < 0.05$; Figure 6B). Concomitant incubation of PA and AA (100 μM) significantly increased insulin secretion at all concentrations of PA used compared with incubation with PA only ($P < 0.0001$; Figure 6B).

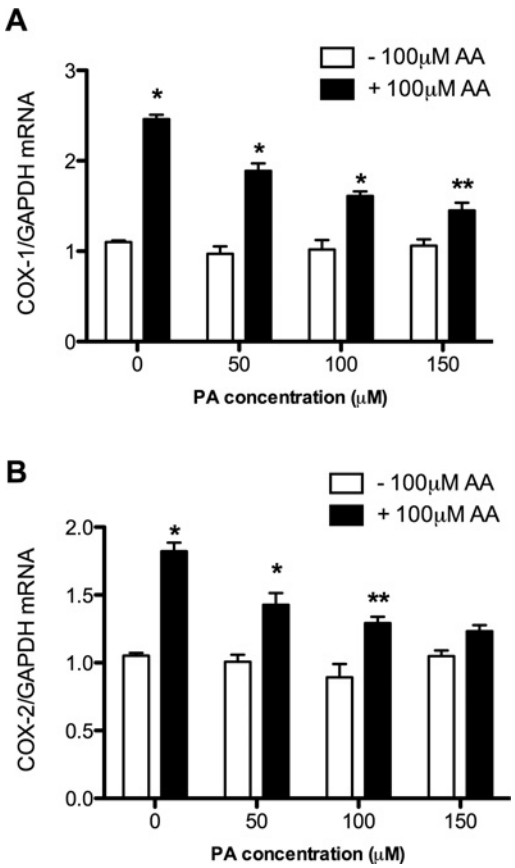

Figure 5 Effect of PA or AA on expression levels of *Cox-1* and *Cox-2*

BRIN-BD11 cells were incubated and in the absence or presence of 100 μM AA and in the absence or presence of increasing concentrations of PA as indicated for 24 h. After this time, the cells were harvested, and the expression levels of *Cox-1* (A) or *Cox-2* (B) were determined by real-time PCR. Expression levels were subsequently normalized to *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) expression. Results are means $\pm$ S.E.M. for three individual experiments. A significant improvement in cell viability (*$P < 0.05$ and **$P < 0.001$) was observed at all concentrations of AA tested.

Influence of PA and AA on oxidative stress

An increase in the production of total ROS and superoxide was observed in BRIN-BD11 cells following incubation with 100 or 150 μM PA for 24 h. Incubation with 100 μM AA alone did not alter either the production of ROS or superoxide when compared with control. AA, however, when added in combination with 100 or 150 μM PA, significantly decreased the level of superoxide and, at 150 μM PA, significantly decreased the level of ROS (Figures 7A and 7B).

Table 2 Rescue of PA-incubated cells by AA as determined by WST-1 absorbance analysis

BRIN BD11 β-cells were incubated in the absence or presence of various concentrations of PA and/or AA at 100 μM for 24 h (50–150 μM PA). Cell viability and proliferation was assessed using an assay based on the reduction of WST-1, a tetrazolium salt, to formazan by cellular mitochondrial dehydrogenase activity and was normalized to 100%. An increase in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. Results are expressed as normalized means $\pm$ S.E.M. for six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.0001$ compared with cell viability from control cells (no fatty acid)

	Relative mitochondrial dehydrogenase activity (%)				
Treatment	Control	PA (μM) . . .	50	100	150
$-$100 μM AA	100.00 $\pm$ 1.1		93.96 $\pm$ 0.5	70.84 $\pm$ 1.86***	58.55 $\pm$ 1.03***
$+$100 μM AA	119.0 $\pm$ 2.75*		98.33 $\pm$ 0.66	83.46 $\pm$ 1.54*	67.56 $\pm$ 5.36**

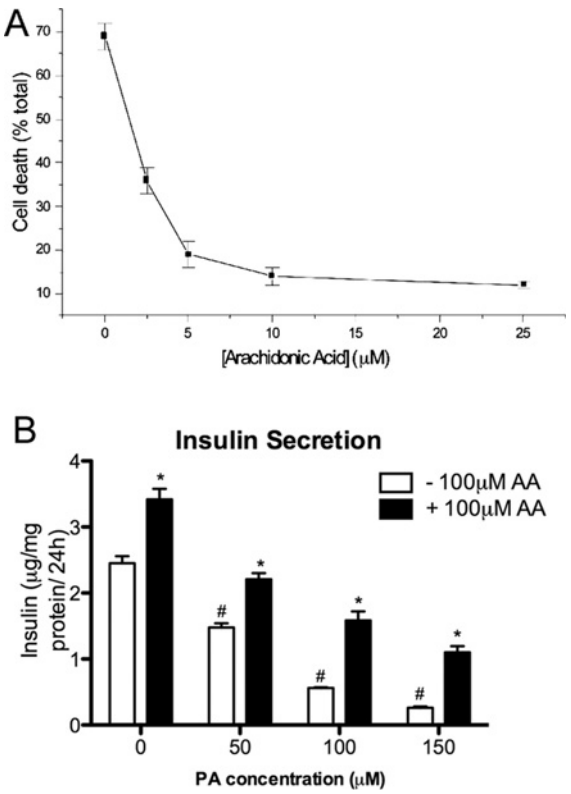

Figure 6 Rescue of PA-incubated cells by AA determined by flow cytometry (A), and effect of PA and AA on chronic (24 h) insulin secretion (B)

(A) BRIN-BD11 cells were incubated in the presence of 250 μM PA complexed to BSA in the absence or increasing concentrations of AA as indicated for 24 h. After this time, the cells were harvested and their viability assessed by flow cytometry after staining with PI. A significant improvement in cell viability ($P < 0.01$) was observed at all concentrations of AA tested. (B) BRIN-BD11 β-cells were incubated in the presence of various concentrations of PA (50–150 μM non-complexed) in the absence or presence of 100 μM AA for 24 h. After the 24-h incubation period, an aliquot of the medium was removed, and the concentration of insulin was determined. The results are expressed as means $\pm$ S.E.M., for three separate determinations each measured in duplicate, relative to cells incubated in the absence of fatty acid ($\#P < 0.05$) or compared with the equivalent PA-only-treated cells ($^{*}P < 0.05$).

Effect of PA and AA on nitrite production

NO was determined by quantifying nitrite, an oxidative product of NO. Cells increased their production of nitrite when incubated with 100 or 150 μM of PA ($P < 0.0001$). However, concomitant incubation of AA and PA decreased the production of nitrite when compared with PA-only-treated cells ($P < 0.001$) (Figure 7C).

Effect of PA and AA on protein expression

PA increased the protein expression of subunit p65 of NF-κB and subunit p47 of NADPH oxidase in a dose-dependent manner. AA, when added concomitantly with PA, decreased the expression of these proteins compared with PA-only-treated cells.

The protein level of the enzyme responsible for NO generation, iNOS (inducible NO synthase), was increased following incubation in the presence of PA. A combination of AA and PA significantly decreased iNOS expression compared with PA-only-treated cells (Figure 8).

Effect of PA and AA on glutathione metabolism

Incubation of cells in the presence of PA resulted in a decrease in intracellular GSH in a dose-dependent manner, while the content of GSSG was increased. AA addition to cells, which were also treated with PA, increased the concentration of GSH and decreased the concentration of GSSG, contributing to an increase in the ratio of GSH/GSSG when compared with the group incubated with PA only (Figure 9).

DISCUSSION

The effects of NEFAs on β-cell function are complex. Pancreatic islets exposed to high concentrations of NEFAs for periods of 24–48 h [3] display enhanced insulin secretion in the presence of a non-stimulatory glucose concentration, decreased insulin synthesis, depletion of stored insulin and an impaired response of

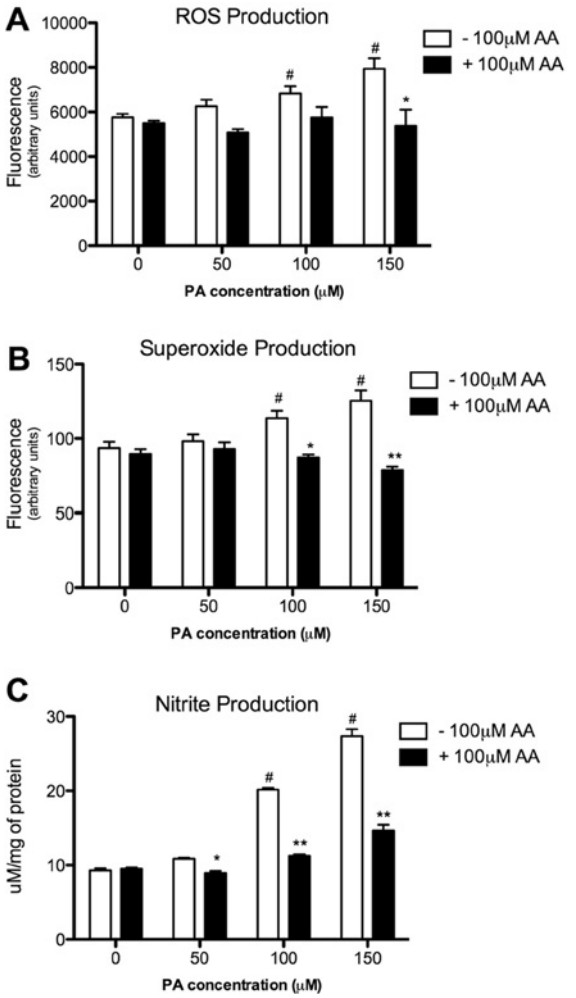

Figure 7 Influence of PA and AA on oxidative stress and nitrite production

BRIN-BD11 cells were incubated for 24 h in the presence of different concentrations of PA and 100 μM AA. (A) ROS production was quantified using the H_2DFCH probe. Results are expressed as means $\pm$ S.E.M., for three independent experiments. (B) Superoxide production was measured using the DHE probe. Results are expressed as means $\pm$ S.E.M., for five independent experiments. $^{\#}P < 0.0001$ compared with the no-fatty-acid control. (C) Nitrite production was measured using the Griess Reagent kit according to the manufacturer's instructions. Results are expressed as means $\pm$ S.E.M., for $n = 5$ independent experiments. $^{\#}P < 0.0001$ compared with the no-fatty-acid control. $^{*}P < 0.0001$ compared with the respective group without 100 μM AA.

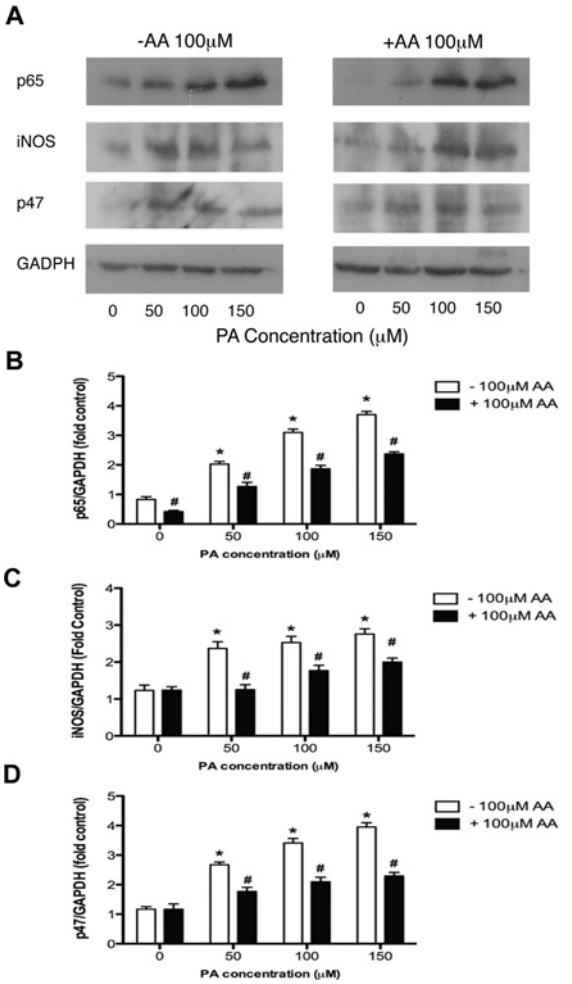

Figure 8 Effect of PA and AA on protein expression

BRIN-BD11 cells were incubated for 24 h in the presence of different concentrations of PA and 100 μM AA. Expression of the p65 subunit of NF-κB, p47 of NADPH oxidase and iNOS was analysed by Western blotting after gel-based protein separation (A). The absorbances of the bands were measured and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein expression (B–D). Results are expressed as means $\pm$ S.E.M., for three independent experiments. $P < 0.05$ compared with cells incubated in the absence of fatty acid; $^{\#}P < 0.05$ compared with the equivalent PA-only-treated cells.

the β-cell to stimulation by high glucose concentrations, all of which are characteristic of the altered β-cell function associated with T2DM. Rat and human islets exposed to elevated levels of NEFAs for 48 h also demonstrated increased basal insulin release (glucose concentration was 3 mM), but decreased release at an elevated (stimulatory) glucose concentration (27 mM) [25].

The effects of high concentrations of NEFA on isolated islets and clonal pancreatic insulin-secreting cells are dependent on the structure (i.e. whether saturated or unsaturated) of the fatty acid and the period of exposure during cell culture. Acute exposure (1–3 h) of pancreatic islets to saturated NEFA enhances insulin secretion [26] and plays a critical role in modulating the stimulatory effect of glucose on insulin release [27]. Acute removal of exogenous NEFA may, however, result in excessively high rates of glucose-stimulated insulin secretion [28]. The latter results may argue in favour of a buffering role for NEFA, such that islet cells utilize some glucose (in the form of a downstream metabolite, glycerol 3-phosphate) for formation of TAG (triacylglycerol), so as to ensure that excessively high rates of insulin secretion do not occur. Expression of UCP-2 in β-cells is increased by NEFAs. UCPs are located in the mitochondrial

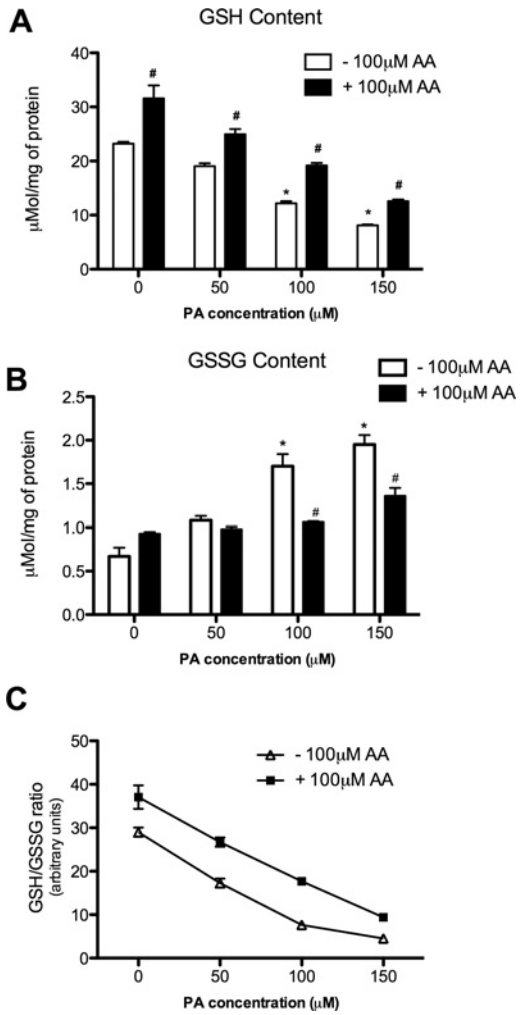

Figure 9 Effect of PA and AA on glutathione metabolism

BRIN-BD11 cells were incubated for 24 h in the presence of different concentrations of PA and 100 μM AA. (A) Intracellular content of GSH was measured. (B) Intracellular content of GSGG was measured. (C) GSH/GSSG ratio. Results are expressed as means $\pm$ S.E.M., for three independent experiments. $^{*}P < 0.0001$ compared with the no-fatty-acid control. In (A and B). $^{\#}P < 0.0001$ compared with the respective group without 100 μM AA.

inner membrane and act as proton channels. They uncouple the electrochemical gradient produced by the respiratory chain from ATP synthesis [29]. Up-regulation of UCP-2 expression or overactivity of UCP-2 leads to inhibition of ATP synthesis and alteration in levels of ROS generation. NEFAs increase UCP-2 mRNA and protein levels in several cells including β-cells. As a result, ATP synthesis is reduced, and GSIS is blunted. *Ucp-2*-knockout mice have lower fasting blood glucose and elevated insulin levels when fed with a high-fat diet compared with WT (wild-type) mice [30]. Exposure to PA reduced GSIS in WT islets, whereas *Ucp-2*$^{-/-}$ islets had enhanced GSIS. While saturated NEFAs may induce detrimental effects on β-cell function in part via increased UCP-2 expression, we clearly demonstrated in the present study (microarray and PCR data) that the polyunsaturated NEFA AA did not increase *Ucp-2* expression, therefore avoiding any UCP-2-dependent deleterious effects associated with saturated NEFAs. Saturated NEFAs can lead to excessive ROS generation from mitochondrial and extramitochondrial sources such as NADPH oxidase in β-cells, accompanied by the loss of antioxidant defence via glutathione depletion [31–33]. Additionally, NO generation in pancreatic β-cells may be stimulated through ligation and signalling via the NEFA receptor GPR40 (G-protein-related protein 40) [34], a possible regulator of iNOS expression [35]. NO competes with molecular oxygen at Complex IV of the electron-transport chain, leading to impaired oxidative phosphorylation and ATP generation, which can induce dysregulation of cellular Ca^{2+} homoeostasis resulting in endoplasmic reticulum stress [36–38].

In contrast with the deleterious effects associated with elevated saturated fatty acids, reports suggest that non-esterified AA is critical for normal pancreatic β-cell function. For example, studies in which the release of endogenous AA was attenuated by inhibiting PLA_2 (phospholipase A_2) activity, revealed a significant reduction in GSIS from human islets [39]. The release of endogenous AA from the plasma membrane via PLA_2-dependent hydrolysis of phospholipids has been identified as an important signalling event [40–42]. Exogenous addition of AA has been reported to augment insulin secretion from pancreatic β-cells [43], which is in agreement with results obtained for chronic (24 h) insulin secretion in the present study (Figure 6B). We have now provided further mechanistic data on the action of AA. The positive effects of AA described here could result from the direct second messenger functions of this molecule, or they could derive from metabolism of AA to biologically active products. Metabolism of non-esterified AA by COX enzymes yields PGs (prostaglandins), while LOX enzymes generate LTs (leukotrienes) and HETEs (hydroxyeicosatetraenoic acids). Previous experiments evaluating the functional effects of PGs (most notably PGE_2) on the β-cell have implicated PGs in the inhibition of glucose-stimulated insulin secretion from a hamster-derived cell line [44]. However, HETEs and LTs produced from LOX enzyme activity augment GSIS [45]. In the present study, we have demonstrated that inhibition of the COX-1 pathway of AA metabolism resulted in an inhibition of D-glucose- and L-alanine-stimulated insulin secretion from BRIN-BD11 cells, in the presence of exogenously added AA. This is consistent with the possibility that COX-2 metabolites (which will be preferentially generated in these conditions) are inhibitory to nutrient-stimulated insulin secretion. In contrast, selective inhibition of COX-2 using NS-398 (100 μM) in combination with 100 μM exogenously added AA significantly ($P < 0.05$) enhanced both

basal- and nutrient-stimulated insulin secretion compared with BRIN-BD11 cells incubated with control media (Table 1). This may indicate that COX-1 metabolites (which will be preferentially generated under these conditions) are stimulatory to basal- and nutrient-stimulated insulin secretion. We also demonstrated that BRIN-BD11 cell COX-1 and COX-2 expression was not altered when incubated with PA. In contrast, AA significantly increased the expression of both enzymes. However, PA was able to reduce enhanced AA expression in a dose-dependent manner. The latter findings correlate well with the insulinotropic effects of addition of both AA and PA over 24 h of incubation (Figure 6B)

The ability of AA to cause down-regulation of *Scd-2* and *Abcg1* and the up-regulation of *Ech1* and *Angptl4* gene expression (Figure 3) would infer a decrease in synthesis of unsaturated fatty acids (*Scd-2*) and a decrease in cholesterol transport to HDL (high-density lipoprotein) (*Abcg1*). An increase in isomerization activity (*Ech1*) would favour the entry of unsaturated fatty acids into the pathway of β-oxidation, whereas an increase in *Angptl4* signals a potential increase in cholesterol synthesis. The expression changes identified by microarray analysis and confirmed with real-time PCR would therefore predict greater levels of unsaturated fatty acid oxidation but a potential increase in cholesterol synthesis and transport in the presence of AA.

The AA-mediated rescue of BRIN-BD11 cells from PA-mediated dysfunction, as reported in the present work, is remarkable and supports earlier data obtained in MIN6 cells [46] This response may be mediated by a reduction in oxidative and inflammatory stress as indicated by reductions in NF-κB, iNOS and NADPH oxidase subunit levels and a reduction in NO and ROS levels respectively, resulting in a corresponding increase in the GSH/GSSG ratio. The positive impact of AA with respect to its effect on cellular oxidative stress is a novel finding and must be considered as a key mechanism of cell protection with respect to fatty-acid-induced β-cell dysfunction. The molecular mechanisms could involve AA- or PG-mediated changes in signal transduction, subsequent gene expression and their resulting impact on redox and inflammatory signalling pathways.

It is clear that further work on the mechanisms of cytoprotection by AA (and/or downstream metabolites) and its ability to modulate insulin secretion is required. This may reveal novel therapeutic options for the treatment of T2DM.

AUTHOR CONTRIBUTION

Philip Newsholme was the principal investigator responsible for this work and takes full responsibility for the manuscript. Deirdre Keane and Hilton Kenji Takahashi completed the majority of experiments described. Shalinee Dhayal completed the experiment described in Figure 6(A). Noel Morgan, Rui Curi and Philip Newsholme co-wrote and revised the manuscript.

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