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Arachidonic acid actions on pancreatic β -cell functional integrity and attenuation of the negative effects of palmitic acid.

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Short title: Arachidonic acid positively regulates β -cell function

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 palmitic acid (PA) are more toxic to β -cells than unsaturated fatty acids such as arachidonic acid (AA). Here, we describe the effect of AA on BRIN-BD11 cell function and demonstrate its ability to attenuate the effect of PA. When added to β -cell incubations at 100uM AA can stimulate cell proliferation and insulin secretion. Microarray analysis indicated significant AA dependent up-regulation of genes involved in proliferation and fatty acid metabolism (e.g. Angptl and ECH1, ($p < 0.05$)). Experiments using specific COX and LOX inhibitors demonstrated the importance of COX-1 activity for stimulation of insulin secretion, suggesting that AA metabolites generated via COX-1 may be responsible for the beneficial effects of AA. Moreover, concomitant incubation of AA with PA does-dependently attenuated the detrimental effects of the saturated FA, so reducing apoptosis and decreasing parameters of oxidative stress (decreasing ROS and NO levels and increasing the GSH/GSSG ratio). AA decreased the protein expression of iNOS, the p65 subunit of NF- κ B and the p47 subunit 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 β -cell function and survival in the 'lipotoxic' environment associated with the development of type 2 diabetes.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by elevated blood glucose and lipid concentrations and decreased glucose-stimulated insulin secretion (GSIS) [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 free fatty acid levels (NEFA) which can lead to reduction in β -cell functional mass (glucolipotoxicity) [3, 4]. NEFAs can inhibit insulin secretion by slowing glucose uptake, metabolism and oxidation via Randle cycle effects, where an increase in availability of NEFA favors 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] and activation of stress related response genes such as NF- κ B [10] could be involved in triggering apoptosis. *In vitro* studies have demonstrated that saturated fatty acids such as palmitic acid (PA) and stearic acid are more toxic than unsaturated fatty acid such as oleic and arachidonic acid (AA) although unsaturated fatty acids are not entirely free of cytotoxic effects at elevated concentrations [11-14]. NEFA, however, in low concentrations are essential for GSIS by

potentiation of glucose-stimulated insulin secretion and can be used as a 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 it 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 24h) 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 nutrient-stimulated insulin secretion. *In vitro* incubation of β -cells with AA can increase glucose- or amino acid- stimulated insulin secretion and also stimulate their proliferation [18]. The metabolism of AA by specific isoforms of cyclooxygenase (COX) generates lipid products that can increase insulin secretion [16]. A recent study showed that concomitant incubation of BRIN-BD11 cells with inhibitors of AA mobilization altered glucose-induced insulin secretion when compared to cells incubated in the presence of AA [19].

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 production and protection from the detrimental effects of PA.

MATERIALS AND METHODS

Reagents

RPMI-1640 media, penicillin/streptomycin, fetal calf serum and glutamine were obtained from Gibco (Paisley, Scotland, U.K.). The WST-1 cell viability assay was obtained from Roche (Roche Diagnostics, Mannheim, Germany). The rat insulin ELISA kit was obtained from Mercodia (Uppsala, Sweden). Griess Reagent System for nitrite detection was obtained from Promega (Madison, WI, USA). All other reagents were obtained from Sigma-Aldrich.

Cell Culture

BRIN-BD11 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 2mM glutamine and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air using a Forma Scientific incubator (Marietta, OH, USA). Cells were kept between 1 x10⁵ and 1x10⁶ cells/ml. For the experiments, cells (1.5x10⁵) were seeded in 24 wells plate or containing 2ml of media or 1.5x10⁶ in 6-well plates containing 5ml of media and allowed to adhere overnight before treatment in the presence or absence of fatty acids. A stock solution of each fatty acid (100mM) was prepared utilising ethanol. The final concentration of ethanol added to the cell culture medium was always less than 0.5 % which showed not to be toxic for the cells (data not shown).

Cell Viability

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. 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 (2x10⁴) were plated in a 96-well plate and let adhere overnight. Cells were treated for 24h and at the end of incubation, the cell proliferation reagent WST-1 (10 μ l) was added to each well. The cells were incubated for 1 hour 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, Sunnyvale, CA, USA). 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 300g for 5 minutes. The pellet was resuspended in 200µl of media. Propidium iodide (PI) staining solution was prepared by mixing 20µg/ml of PI with FACS buffer (PBS, 2% FCS and 10mM sodium azide). 200µl of this PI solution was then added to the samples and incubated on ice for 10 minutes. 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 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 Krebs Ringer buffer (KRB) pH 7.4 containing 1.1mM D-glucose, followed by subsequent stimulation for 20 min in KRB pH 7.4 containing 16.7mM glucose plus 10mM 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 (Uppsala, Sweden).

RT-PCR

Total RNA was isolated from cells using TRI REAGENT (Molecular Research Center, Inc.) according to standard protocols provided by the manufacturer. cDNA synthesis was carried out on the total RNA using the Superscript Pre-amplification System II (Invitrogen Life Technologies Paisle, Scotland, U.K.). The primers used were SREBP-1c (forward primer, *tcagttcvcagcatggctacc*; reverse primer, *gcagttgatgtagaggctaa*), PPAR-α (forward primer, *ggccgattcttccactgc*; reverse primer, *tcccctctgcaacttctc*), PPAR-δ (forward primer, *gcaggctctagaattccatc*; reverse primer, *gccgtgtctggagtgtgtg*), AMPK (forward primer, *tgctttgctgtgtggaactc*; reverse primer, *tggtgtacaggcagctgagg*), GCK (forward primer, *cagtgagcgtgaagacaaa*; reverse primer, *accatccggctacataccag*), UCP-2 (forward primer, *attgcacgagaggaagggat*; reverse primer, *attactacgttcaggatcc*), IR (forward primer, *cattcaggaagaccttcgagg*; reverse primer, *tgcaatcaggactccccaga*). Expression of genes of interest was normalised to 18S rRNA amplified from the same samples and its expression levels were not altered by any treatment reported in the present study.

Microarray

Total RNA from the cells of was isolated using RNeasy kit (Qiagen, Valencia, CA, USA). RNA samples were prepared for microarray analysis using the GeneChip System (Affymetrix Inc, Santa Clara, CA, USA). 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-labeled cRNA was then prepared using the GeneChip IVT Labelling kit. Subsequently the biotin-labeled cRNA was cleaned using the GeneChip Sample Cleanup module and following quantitation was fragmented at 94 °C prior to hybridisation with Rat Genome 230 2.0 Array. After hybridisation, 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, Inc.) according to the manufacturer's protocol. cDNA synthesis was carried out on the total RNA using the Superscript Pre-amplification System II (Invitrogen Life Technologies Paisley, Scotland, U.K.). Real-Time PCR TaqMan assay was used to quantitate 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 normalisation 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/delta ct method of analysis.

Reactive Oxygen Species (ROS) Detection

After incubation cells were washed with PBS and lysed with lysis buffer (1% Triton-X, 40mM EDTA). Lysates were then transferred to a 96-wells opaque plate and incubated for 30 min with 10µM 2',7'-di-hydrochlorofluorescein-diacetate (H₂DCFDA) (Molecular Probes). Its oxidation by ROS releases DCF which is fluorescent (Ex/Em 495/527 nm). Fluorescence was measured in a Spectramax Plus Fluorescence Plate Reader (Molecular Devices, Sunnyvale, CA, USA).

Superoxide Production

Cells were seeded in an opaque 96-well plate and after treatment, 10µM of dihydroethidine (DHE) 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, Sunnyvale, CA, USA).

Nitrite Production

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

Intracellular Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) Measurement

Intracellular GSH and GSSG content was determined using a protocol described by Rahman, et al (2007) [20]. The assay is based on the reaction of GSH with DTNB (also known as Ellman's reagent) that produces the TNB chromophore, which has a maximal absorbance at 412 nm, and oxidized glutathione–TNB adduct (GS–TNB). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample. The disulfide product (GS–TNB) is then reduced by GR in the presence of NADPH, recycling GSH back into the reaction.

Briefly, cells were lysed in a extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in 0.1M potassium phosphate buffer with 5mM EDTA disodium salt, pH 7.5) for 15 min under agitation and centrifuged at 12000rpm for 10min. The supernatant was collected and stored at -80C. An aliquot was kept for protein measurement.

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

For GSSG measurement, samples and GSSG standards were incubated with 2-vinylpyridine and triethanolamine for 1 hour at room temperature and then transferred to a 96-well plate with RM and read at 412nm. Results were expressed as $\mu\text{g}/\text{mg}$ of protein.

Western blot analysis

After the incubation period, cells were trypsinized and centrifuged at 1200 rpm for 10 minutes and homogenized in extraction buffer (100 mM Tris-HCl, pH 7.0; 10 mM EDTA; 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate). The samples were sonicated and SDS (10%) was added followed by 30 minutes of incubation on ice. After that, samples were centrifuged at 12000 rpm, for 40 min, at 4°C. Aliquots of supernatants were used for measurement of total protein content as described by Bradford [21]. Equal amounts of protein from each sample were separated using SDS–polyacrylamide gel electrophoresis. Western blotting was carried out following the method described by Towbin et al. [22]. Proteins of the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. After the protein transfer the membrane was placed in Millipore SNAP id Protein Detection System (Millipore, USA). The membranes were blocked using 0.3% defatted milk in TBS-T buffer (10 mM Tris-HCl, pH 7.0; 150 mM NaCl; 0.05% Tween 20). Primary incubation involved incubation in 1:5000 dilution of p65 antibody, 1:1000 dilution of p47phox or 1:10000 dilution iNOS antibodies for 10 minutes. Secondary incubation of the membrane was then performed. The bands were developed by detection using Pierce ECL western blot substrate kit (Pierce, Rockford, IL) and immediately exposed to X-ray film for 1–5 min. Films were then revealed in the conventional manner.

Statistical analysis

The results are presented as mean \pm standard error of mean (S.E.M.) and analyzed 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 cell viability

Incubation of BRIN BD11 cells with AA (up to 150 μM) for 24h did not impair cell viability when compared to 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 functional mitochondria. The addition of 100 μM of AA however, stimulated cell proliferation increasing the cell population by 19% when compared to control. Incubation of the BRIN BD11 cells with a relatively high level of 150 μM AA resulted in a decrease in cell viability when compared to cells incubated with 100 μM , but not when compared to vehicle control (Figure1).

Effect of AA on acute (20min) insulin secretion

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

Effect of AA on modulation of gene expression

Representative gels of RT-PCR analysis comparing SREBP-1c, PPAR- δ , PPAR- α , insulin, UCP-2, AMPK and GCK mRNA expression in BRIN BD11 cells treated in the absence of or increasing concentrations of AA for 24 hours are described in Figure 3. There was a significant (** $p < 0.01$) decrease in relative expression of SREBP-1c following 24hour exposure to 50 or 100 μM AA compared to cells cultured in control media (Figure 3A). The relative expression levels of

PPAR- α mRNA in cells treated with 40, 50 or 100 μ M AA was significantly (* $p < 0.05$, ** $p < 0.01$) reduced compared with control cells (Figure 3B). There was also a significant (* $p < 0.05$) reduction in relative expression of PPAR- δ mRNA in BRIN BD11 β -cells stimulated for 24 hours with 50 or 100 μ M AA (Figure 3C). There was no significant change in insulin, UCP-2 or GCK mRNA expression following 24 hour stimulation with AA (Figure 3D, E and G). However, a significant (* $p < 0.05$) increase in the relative expression of AMPK mRNA was seen in BRIN BD11 cells stimulated for 24 hours with 100 μ M AA, compared to untreated control cells (Figure 3F).

Microarray analysis and subsequent data analysis (using freeware from the Bioconductor website (www.bioconductor.org)) revealed that 4 genes were significantly altered in response to chronic (24 hours) treatment of BRIN BD11 cells with 100 μ M AA (Table 1). The down regulation of SCD-2 and Abcg1 observed using microarray analysis was confirmed using real time PCR (Figure 4 A and B). The up-regulation of Ech1 and Angptl4 (microarray analysis) was also confirmed using real time PCR (Figure 4 C and D).

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

In order to determine whether the effects of AA on β -cell viability and function were mediated by the products of AA metabolism or by AA itself, specific cyclooxygenase (COX) enzyme inhibitors and a non selective lipooxygenase (LOX) enzyme inhibitor were used (Figure 5).

Selective inhibition of COX-1 by 100 μ M acetaminophen decreased significantly ($p < 0.05$) the extent of nutrient stimulated insulin secretion in the presence of 100 μ M AA. This finding suggests that the inhibition of the COX-1 pathway results in a loss of the potentiating effect of exogenous AA on D-glucose and L-alanine stimulated insulin secretion. No differences were found in basal insulin secretion. Inhibition of COX-1 with 10 μ M 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) in combination with 100 μ M AA, however, significantly (* $p < 0.05$) enhanced insulin secretion measured under non-stimulating conditions compared to control. There was no significant effect on nutrient stimulated insulin secretion from cells incubated with SC-560 and AA.

Combinations of 100 μ M indomethacin (a non-specific COX and LOX inhibitor) with 100 μ M AA significantly reduced basal (** $p < 0.01$) and nutrient ($^{##}p < 0.01$) stimulated insulin secretion when compared to control.

Selective inhibition of COX-2 via 100 μ M N-(2-cyclohexyloxy)-4-nitrophenylmethanesulfonamide (NS-398) in the presence of 100 μ M AA significantly enhanced basal (* $p < 0.05$) and nutrient stimulated ($^{##}p < 0.01$) insulin secretion when compared to 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 μ M AA alone.

Rescue of PA incubated cells with AA

Incubation of BRIN BD11 cells with PA for 24h decreased the cell viability in a dose dependent manner as determined by WST-1 analysis ($^{#}p < 0.001$). However, concomitant incubation with AA protected BRIN BD11 cells from the toxic effect of PA in a dose-dependent manner as judged either by WST-1 analysis (Table 2) or by flow cytometry (Figure 6A). The latter revealed that the cytoprotective response was extremely potent with an $EC_{50} \sim 2\mu$ M under the experimental conditions used and that AA caused complete protection against the cytotoxicity induced by 250 μ M PA.

Effect of PA and AA on chronic (24h) insulin secretion

PA decreased cumulative insulin secretion over a 24h incubation period in a dose dependent manner ($p < 0.05$, Figure 6B). Concomitant incubation of PA and AA (100 μ M) significantly increased insulin secretion at all concentrations of PA used compared to incubation in PA only ($p < 0.0001$, Figure 6B).

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 μ M or 150 μ M of PA for 24h. Incubation with 100 μ M AA alone did not alter either the production of ROS or superoxide when compared to control. AA however, when added in combination with 100 μ M or 150 μ M PA, significantly decreased the level of superoxide and at 150 μ M PA significantly decreased the level of ROS (Figure 7 A and 7B).

Effect of PA and AA on nitrite production

Nitric oxide was determined by quantifying nitrite, an oxidative product of NO. Cells demonstrated increased production of nitrite when incubated with 100 and 150 μ M of PA (#p<0.0001). Concomitant incubation of AA and PA decreased the production of nitrite when compared to palmitic only treated incubated cells (**p<0.001) (Figure 7C).

Effect of PA and AA on protein expression

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

The protein level of the enzyme responsible for NO generation, iNOS, was increased following incubation in the presence of PA. Combination of AA and PA did not result in decreased iNOS expression. (Figure 8)

Effect of PA and AA on Glutathione metabolism

Incubation 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 increase the ratio of GSH/GSSG when compared to 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 NEFA for periods of 24–48 h [3, 23] display enhanced basal insulin secretion, decreased insulin synthesis, depletion of stored insulin, and an impaired response of the β -cell to stimulation by glucose; all of which are characteristics seen in type 2 diabetes. Rat and human islets exposed to elevated levels of fatty acids for 48 h demonstrated increased insulin release at basal glucose concentration (3mM) but decreased release at an elevated (stimulatory) glucose concentration (27mM) [24].

The effects of high concentrations of NEFA on isolated islets and clonal pancreatic insulin secreting cells are dependent on the saturated/unsaturated nature 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 [25] and plays a critical role in modulating the stimulatory effect of glucose on insulin release [26]. Acute removal of exogenous NEFA may however, result in excessively high rates of glucose-stimulated insulin secretion. After incubation of rat pancreatic islets for 4 h with a high concentration of fatty acid-free BSA they responded to glucose with extraordinarily high rates of secretion, without a change in the typical biphasic pattern of the response [27]. The latter results may argue in favor of a buffering role for NEFA, such that islet cells utilize some glucose (in the form of a down-stream metabolite, glycerol 3-phosphate) for formation of Triacylglycerol (TAG) so as to ensure that excessively high rates of insulin secretion do not occur. TAG stores may subsequently release NEFAs on activation of specific TAG lipases.

Expression of uncoupling protein 2 (UCP-2) in β -cells is increased by NEFAs. UCPs are located in the inner mitochondrial membrane and act as proton channels. They uncouple the electrochemical gradient produced by the respiratory chain from ATP synthesis [28, 29].

Upregulation of UCP-2 expression or overactivity of UCP-2 leads to inhibition of ATP synthesis and alteration in levels of ROS generation. Fatty acids 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 as compared with WT (wild-type) mice [30]. Exposure to palmitate reduced GSIS in WT islets, whereas UCP2 (-/-) islets had enhanced GSIS [31]. While saturated NEFAs may induce detrimental effects on β -cell function in part via increased UCP-2 expression, we clearly demonstrated in this work (microarray and PCR data) that the polyunsaturate, AA, did not increase UCP-2 expression; thus, possibly avoiding the deleterious effects associated with saturated NEFAs. NEFA and hyperglycemia can lead to excessive ROS generation from mitochondrial and extra-mitochondrial sources such as NADPH oxidase accompanied by the loss of antioxidant defense via glutathione depletion [32, 33]. Also, there may be an elevated level of NO generation in pancreatic β -cells through ligation and signaling via the NEFA receptor GPR40, a possible regulator of iNOS expression [34, 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 $[Ca^{2+}]$ homeostasis resulting in endoplasmatic reticulum stress [36-38].

In contrast to chronic exposure to saturated fatty acids, reports suggest that non-esterified AA is critical for normal pancreatic β -cell function. Studies in which the release of endogenous AA was attenuated by inhibiting PLA2 activity, revealed a significant reduction in GSIS from human islets [39]. With respect to intracellular factors that may regulate GSIS from the β -cell, the release of endogenous AA from the plasma membrane via PLA₂ hydrolysis of phospholipids has been identified as an important event [40-42]. Additionally, exogenous AA has been reported to augment insulin secretion from pancreatic β -cells [43, 44].

The positive effects of AA on attenuating the negative effects of palmitic acid on chronic (24h) insulin secretion could be the result of its own actions as a second messenger or they could result from metabolism of AA to biologically active products. Metabolism of non-esterified AA by COX enzymes yields prostaglandins (PGs) while LOX enzymes generate leukotrienes (LTs) and hydroxyecosatetraenoic acids (HETEs) [45]. Previous experiments evaluating the functional effects of PGs (most notably PGE₂) on the β -cell have implicated these lipid mediators in the inhibition of glucose stimulated insulin secretion [46, 47]. However, initial reports demonstrated that the HETEs and LTs produced from LOX enzyme activity augmented glucose stimulated insulin secretion [48-50]. Surprisingly, more recent experiments have implied that PGE₂ may not inhibit insulin secretion from human islets [45].

Importantly, few studies have attempted to explore the interplay between glucose, amino acids and fatty acids with respect to β -cell mass and functional integrity *in vitro*. In a previous study, culture of clonal BRIN BD11 β -cells for 24 h with the polyunsaturated fatty acid, arachidonic acid (AA) increased β -cell proliferation and enhanced amino acid (alanine) stimulated insulin secretion [18]. Conversely 24 h exposure to the saturated fatty acid, palmitic acid (PA) was found to decrease β -cell viability (by increasing apoptosis), increase the intracellular concentration of triglyceride, while inhibiting alanine-stimulated insulin secretion [18]. We have now expanded on these studies by probing the effects of exogenously added AA on parameters of cellular function including insulin secretion in the presence of COX1 or COX2 inhibitors. Here 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 may indicate 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 μ M) in combination with 100 μ M exogenously added AA significantly ($p < 0.05$) enhanced both basal and nutrient stimulated insulin secretion compared to BRIN BD11 cells incubated with control media (Table 1). This may indicate that COX-1 metabolites (which will be preferentially generated in these conditions) are stimulatory to basal and nutrient stimulated insulin secretion.

The AA mediated rescue of BRIN BD11 cells from PA mediated dysfunction is remarkable and may be mediated by a reduction in oxidative and inflammatory stress as indicated by changes in NF- κ B and NADPH oxidase subunit expression and a reduction in ROS levels. The molecular mechanisms could involve PG mediated changes in signal transduction and their resulting impact on redox and inflammatory signaling pathways although they might also be mediated independently of AA metabolism since, in unpublished work, we have noted that the poorly metabolised methyl-ester of AA is also cytoprotective

It is clear that further work on the mechanisms of AA (and metabolites) modulation of insulin secretion in the β -cell is required. This may reveal novel therapeutic options for the treatment of Type-2 diabetes.

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Abbreviations

AA, aracidonic acid; NEFA, Non-esterified fatty acid; COX, cyclooxygenase; LOX, lipoxygenase; LC acyl CoA, Long chain acyl CoA; TAG, Triacylglycerol; DAG, Diacylglycerol; HSL, Hormone sensitive lipase; GSIS, Glucose stimulated insulin secretion; UCP-2, uncoupling protein 2.

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Table 1

Function	Accession No	Gene Name	Fold Change	p Value
Lipid Metabolism	NM_031841	Stearoyl-Coenzyme A desaturase 2 SCD-2	-3.87	0.0018
Cholesterol transport/lipid trafficking	NM_053502	ATP binding cassette transporters ABCG1	-1.7	0.0284
Angiogenesis/differentiation	AA818262	Angiopoietin like 4 Angptl-4	+2.72	0.0134
Fatty acid metabolism mitochondrial and peroxisomal	NM_022594	Enoyl-Coenzyme A hydratase 1 ECH1	+1.73	0.0284

Table 2

	Control	PA 50 μ M	PA 100 μ M	PA 150 μ M
- 100 μ M AA	100.10 \pm 1.1	93.96 \pm 0.5	70.84 \pm 1.86 [#]	58.55 \pm 1.03 [#]
+ 100 μ M AA	119.0 \pm 2.75*	98.33 \pm 0.66	83.46 \pm 1.54*	67.56 \pm 5.36**

Figure and Table Legends

Figure 1: BRIN BD11 β -cells were incubated in the absence (Ctrl) or presence of various concentrations of AA for 24 hours (50 - 150 μ M AA). 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. An increase in the number of viable cells results in an increase in the overall mitochondrial dehydrogenase activity. Results are expressed as mean \pm SEM, for 6 independent experiments. * p < 0.05 compared to basal insulin secretion from control cells.

Figure 2: BRIN BD11 β -cells were incubated in the absence (Ctrl) or presence of 100 μ M AA for 24 hours. Subsequently an acute (20 minute) determination of basal (1.1mM glucose) and nutrient (16.7mM glucose and 10mM alanine) stimulated insulin secretion was obtained. Results are expressed as mean \pm SEM, for 6 independent experiments. * p < 0.05 compared to basal insulin secretion from control cells.

Figure 3: BRIN BD11 β -cells were treated in the absence (Ctrl) or presence of 25, 40, 50 or 100 μ M AA for 24 hours. The agarose gels shown (H - N) are each representative of 4 independent experiments, each condition was analysed in duplicate. The graphs (A - G) of SREBP-1c, PPAR- δ and PPAR- α , Insulin, UCP-2, AMPK and GCK mRNA expression are relative to control (18 S rRNA) gene expression. The values represent means \pm SEM. * p < 0.05 ** p < 0.01 compared to cells incubated in the absence of any FA.

Figure 4: BRIN BD11 β -cells were cultured in the absence (Ctrl) or presence of 100 μ M AA for 24 hours. Expression of A) Scd2, B) ABCG-1, C) ECH1, D) Angptl4 was determined by real time PCR and normalized to 18S rRNA amplified from the same samples. Results are mean \pm S.E.M. of three individual experiments where each condition was performed in duplicate. The relative ratio for the control cells were arbitrarily set to 1. * p < 0.05, ** p < 0.01 compared to control cells.

Figure 5: BRIN BD11 β -cells were incubated in the absence (Ctrl) or presence of 100 μ M AA or 100 μ M AA in combination with 100 μ M acetaminophen or 100 μ M indomethacin, or 100 μ M NS-398 or 10 μ M SC-560 for 24 hours. After which an acute (20 minutes) determination of basal and nutrient (D-glucose and L-alanine) stimulated insulin secretion was obtained. Results are expressed as mean \pm SEM, for 6 independent experiments. * p < 0.05, ** p < 0.01 compared to cells incubated with control media. # p < 0.05, ### p < 0.01 compared to cells incubated with 100 μ M AA and stimulated with 16.7 mM D-glucose and 10 mM L-alanine.

Figure 6A: BRIN-BD11 cells were incubated in the presence of 250 μ M PA plus increasing concentrations of AA as shown for 24h. After this time the cells were harvested and their viability assessed by flow cytometry after staining with propidium iodide. A significant improvement in cell viability (P < 0.01) was observed at all concentrations of AA tested.

Figure 6B: BRIN BD11 β -cells were incubated in the presence of various concentrations of PA (50 -150 μ M) in the absence (Ctrl) or presence of 100 μ M AA for 24 hours. After the 24 hour incubation period, an aliquot of the medium was removed and the concentration of insulin was determined. The results are expressed as mean \pm SEM, for 3 separate determinations each measured in duplicate, relative to cells incubated in the absence of fatty acid (p < 0.05), or compared to the equivalent PA only condition cells (* p < 0.05).

Figure 7: BRIN-BD11 cells were incubated for 24 hours in the presence of different concentrations of PA and 100 μ M AA. In A, ROS production was quantified using the DFCH probe. Results are expressed as mean \pm SEM, for n = 3 independent experiments. # p < 0.0001 as compared

to control. * $p < 0.0001$ as compared to respective group without 100uM AA. In B, superoxide production was measured using the DHE probe. Results are expressed as mean \pm SEM, for $n = 5$ independent experiments. # $p < 0.0001$ as compared to control. * $p < 0.0001$ as compared to respective group without 100uM AA. In C nitrite production was measured using the Griess Reagent kit probe. Results are expressed as mean \pm SEM, for $n = 5$ independent experiments. # $p < 0.0001$ as compared to control. * $p < 0.0001$ as compared to respective group without 100uM AA.

Figure 8: BRIN-BD11 cells were incubated for 24 hours in the presence of different concentrations of PA and 100uM AA. Expression of p65, p47 and iNOS was analyzed by western blotting after gel based protein separation. Optical densities of the bands was measured and normalized to GAPDH protein expression. Results are expressed as mean \pm SEM, for $n = 3$ independent experiments.

Figure 9: BRIN-BD11 cells were incubated for 24 hours in the presence of different concentrations of PA and 100uM AA. In A, intracellular content of reduced glutathione (GSH) was measured. Results are expressed as mean \pm SEM, for $n = 3$ independent experiments. # $p < 0.0001$ as compared to control. * $p < 0.0001$ as compared to respective group without 100uM AA. In B, intracellular content of oxidized glutathione (GSSG) was measured. Results are expressed as mean \pm SEM, for $n = 3$ independent experiments. # $p < 0.0001$ as compared to control. * $p < 0.0001$ as compared to respective group without 100uM AA. In C, the GSH/GSSG ratio was calculated and the result was plot in a graph. Results are expressed as mean \pm SEM, for $n = 3$ independent experiments. # $p < 0.0001$ as compared to control. * $p < 0.0001$ as compared to respective group without 100uM AA.

Table 1: List of BRIN BD11 β -cell genes differentially regulated >1.7 fold following 24 hour incubation with 100 μ M AA compared to control (- NEFA treated) cells.

Table 2: BRIN BD11 β -cells were incubated in the absence (Ctrl) or presence of various concentrations of PA and/or AA at 100 μ M for 24 hours (50 - 150 μ M 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. 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 mean \pm SEM, for 6 independent experiments. * $p < 0.05$ compared to basal insulin secretion from control cells.



















