Anti-Apoptotic Effects of Arachidonic Acid and Prostaglandin E₂ in Pancreatic β -Cells

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Key Words

β -cells • Caspase-3 • Apoptosis • Arachidonic acid • Prostaglandin E₂

Abstract

Background/Aims: The polyunsaturated fatty acid arachidonic acid (AA) has been implicated in β -cell defence mechanisms and prostaglandin (PG) products of cyclooxygenase (COX) 2 action confer resistance to alloxan-induced apoptosis in insulin-secreting RIN cells. We have now investigated the anti-apoptotic effects of AA and its metabolite, PGE₂, in the MIN6 mouse insulin-secreting β -cell line and mouse islets. **Methods:** Apoptosis was determined in MIN6 β -cell and mouse islet extracts by measurement of caspase-3 activity, and COX2 mRNA levels were quantified by real-time RT-PCR. **Results:** Exposure of MIN6 cells to AA (3.1–12.5 μ M) caused concentration-dependent reductions in apoptosis, and similar results were obtained when endogenous AA levels were elevated in cytosolic phospholipase A₂-overexpressing MIN6 cells. 25mM glucose caused both a significant up-regulation of MIN6 cell COX2 mRNA levels and a decrease in apoptosis. Inhibition of MIN6 cell COX2 activity with a selective inhibitor,

NS-398 (10–100 μ M), increased apoptosis and exogenous PGE₂ (0.2–5 μ M) reduced NS-398-induced apoptosis in a concentration-dependent manner. The protective effects of AA and PGE₂ were also observed in primary mouse islets. **Conclusion:** These data show that AA and its COX2-generated metabolite, PGE₂, can protect β -cells from apoptosis.

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Introduction

Arachidonic acid (AA) is an ω -6 polyunsaturated fatty acid that acts as a precursor lipid for the generation of prostaglandins (PGs) by cyclooxygenase isoenzymes (COX1 and COX2) and hydroxyeicosatetraenoic acids (HETEs) and leukotrienes by lipoxygenase (LOX) enzymes. Saturated fatty acids such as palmitate and stearate have been implicated in pro-apoptotic signalling cascades [1–3] and although it has also been reported that unsaturated fatty acids such as AA can also induce apoptosis in some cell types [4, 5], other studies suggest

that AA protects against apoptosis [2, 6]. There is convincing evidence that overexpression of COX2 decreases apoptosis [7] and COX-generated PG products are also reported to be anti-apoptotic in a number of cell types [8-10]. Cellular levels of AA and PGs depend on the relative expression and activities of phospholipase A₂ (PLA₂) enzymes that are responsible for AA hydrolysis from membrane phospholipids and COX enzymes that catalyse the rate-limiting step in PG synthesis by converting AA into PGH₂.

It is generally accepted that the development of Type 2 diabetes is associated with a gradual loss of insulin-secreting β -cells in the islets of Langerhans and this reduction in β -cell mass is caused by perturbations in the balance between β -cell apoptosis and proliferation (reviewed in [11]). There is some circumstantial evidence that AA and/or PGE₂ may be important in regulating this balance. Thus, glucose stimulates both AA generation [12, 13] and up-regulation of COX2 and PGE₂ levels [13-16] in islets, and it also protects against apoptosis in rat β -cells [17] and the MIN6 β -cell line [18]. Given the reported reductions in apoptosis in response to prostaglandins in a variety of cell types [8-10, 19-21] it is possible that glucose-stimulated AA generation and metabolism may be responsible, at least in part, for the anti-apoptotic effects of glucose in β -cells.

To date there have been few studies directly investigating the effect of AA or PGE₂ on β -cell apoptosis, although there is some evidence to suggest that they may both play protective roles. Thus, both AA and PGE₂ suppressed alloxan-induced cytotoxicity in RIN insulinoma cells in vitro and prevented alloxan-induced diabetes in vivo [22-25]. In addition, deletion of cPLA₂ expression in the non obese diabetic mouse model of Type 1 diabetes resulted in severe insulinitis and an increased incidence of diabetes, consistent with cPLA₂-mediated AA release playing a protective role in the pathogenesis of autoimmune diabetes [26]. These observations provide circumstantial evidence for anti-apoptotic roles for AA and/or PGE₂ in β -cells, but direct measurements of activated apoptotic pathways were not made in these earlier studies.

In this study we have investigated the effects of AA, COX2 inhibition and PGE₂ on β -cell apoptosis by measuring caspase-3 activity in MIN6 β -cells and primary mouse islets. We show for the first time that AA metabolism to PGE₂ by COX2 protects β -cells from undergoing apoptosis. These data suggest that AA metabolism is a key target for manipulating the β -cell apoptotic events experienced in Type 2 diabetes.

Material and Methods

Materials

MIN6 cells were kindly provided by Dr. Y. Oka and Professor J. Miyazaki (then at University of Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), PBS, penicillin/streptomycin, L-glutamine, trypsin-EDTA, ethidium bromide, collagenase type XI, Histopaque-1077, AA, N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide (DEVD) and PGE₂ were purchased from Sigma-Aldrich (Dorset, UK). NS-398 and baicalein were from Calbiochem (Nottingham, UK). REV5901 and methyl arachidonyl fluorophosphonate (MAFP) were obtained from Alexis Corporation UK limited, (Nottingham, UK) and cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC) was obtained from Biomol International (Exeter, UK). CoomassieR Plus Protein Assay Reagent Kit was from Perbio Science UK Ltd. (Northumberland, UK). Fetal bovine serum (FBS) and RPMI 1640 were from Invitrogen (Paisley, UK). RNeasy mini kits, PCR primers and gel extraction kits were obtained from Qiagen (West Sussex, UK). Standard PCR was carried out using a Px2 Thermal RT-PCR cycler (Thermo Scientific, Epsom, UK) and real-time quantitative PCR was performed using a LightCycler rapid thermal cycler system from Roche Diagnostics (Sussex, UK).

Mouse islet isolation

Islets were isolated by collagenase digestion of mouse pancreas followed by purification on a Histopaque gradient, essentially as described previously [27]. Briefly, mice were killed by cervical dislocation and 2-3ml cold collagenase solution (1mg/ml) was injected into the pancreas via the common bile duct. The distended pancreas was removed and incubated at 37°C for 10 minutes after which the digested pancreatic tissue was subjected to density gradient separation using Histopaque-1077. Islets were maintained in culture overnight in RPMI 1640 then serum and glucose-deprived for 5 hours (DMEM, 2.5mM glucose, no serum) before 48 hour incubations for apoptosis experiments.

Cell culture

MIN6 insulin-secreting cells (passage 25-35) were maintained in culture in DMEM supplemented with 2mM glutamine, 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. For apoptosis and gene expression measurements cells at approximately 60-70% confluence in 60mm dishes were serum- and glucose-deprived for 24 hours (DMEM, 2.5mM glucose, no serum), followed by a 48 hour incubation under the experimental conditions described in the Results section. Some experiments were performed using MIN6 cells in which cPLA₂ had been stably overexpressed [28]. Transfected cells were maintained in medium supplemented with 1mg/ml G418. Passage matched, non-transfected MIN6 cells were used as controls because these cells are phenotypically similar to MIN6 cells stably transfected with empty pcDNA3.1 [29].

Caspase-3 assay

Apoptosis in MIN6 cells and mouse islets was determined by measuring caspase-3 activity. Kill curves were constructed for all inhibitors and AA metabolites used in these

Fig. 1. Effects of AA on MIN6 cell apoptosis: MIN6 cells were exposed to 2.5mM and 17mM glucose for 48 hours in the absence or presence of AA (3.1-12.5 μ M), and apoptosis was assessed by measuring caspase-3 activity. AA significantly ($P<0.05$) reduced apoptosis at 2.5mM glucose, but had no effect at 17mM glucose. Data are means $\pm$ SEM, $n=3$.

experiments to ensure that the concentrations used were not toxic over a 48 hour incubation period in the absence of serum. Serum-deprived cells/islets were maintained in culture for 48 hours in the presence of 2.5mM glucose in the absence or presence of AA or PGE₂, and other agents as described in the Results section. At the end of the treatment period, the medium was removed and MIN6 cells and islets were retrieved in cell lysis buffer (CLB: 100mM HEPES, pH 7.4, 100mM NaCl, 0.1% (v/v) NP-40, 19mM dithiothreitol, 500 μ M Tris-EDTA). Islets were briefly sonicated (10s, 2 μ) and MIN6 cell and islet lysates were centrifuged (12000g, 5min, 4°C). Protein content of the lysates was quantified by the Bradford assay. Lysates from 50-70 μ g of MIN6 cell or islet protein were diluted in CLB and an equivalent volume of DEVD (final concentration 200 μ M) mixed with assay buffer (CLB supplemented with 20% (v/v) glycerol) was added to each protein lysate. The degree of apoptosis was assessed by measuring increases in caspase-3 activity by spectrophotometric detection at 405nm of cleavage of the chromophore p-nitroanilide from DEVD.

Quantitative RT-PCR

Serum- and glucose-deprived MIN6 cells were maintained in culture in the presence of 2.5 or 25mM glucose for 6, 24 and 48 hours, and RNA was extracted and reverse transcribed essentially as described previously [29]. COX2 mRNA levels were quantified by real-time RT-PCR amplification using a LightCycler rapid thermal cycler system and expressed relative to β -actin mRNAs in the same samples. Primers for mouse COX2 and β -actin mRNAs were as follows: COX2 sense: 5'-TTC AAA AGA AGT GCT GGA AAA GGT-3'; COX2 antisense: 5'-GAT CAT CTC TAC CTG AGT GTC TTT-3'; β -actin sense: 5'-ACG GCC AAG TCA TCA CTA TTG-3'; and β -actin antisense: 5'-AGC CAC CGA TCC ACA CAG A-3'. The predicted sizes of COX2 and β -actin PCR products were 304 and 300bp, respectively. Real-time PCR was performed in a 10 μ l volume as described previously [29]. All PCR protocols included a 10s denaturation step and then continued for 45 cycles consisting of a 95°C denaturation step, annealing for 10s at 52°C (COX2) or 58°C (β -actin) and a 72°C extension phase for 12s. Fluorescence meas-

urements were taken at 83°C (β -actin) and 85°C (COX2) for 2s to eliminate fluorescence from primer-dimer formation. The amplification products were subjected to melting point analyses and subsequent gel electrophoresis to ensure specificity of amplification. Reaction mixtures were separated on 1.8% agarose gels, and ethidium bromide (0.5 μ g/ml) was used to visualise the products.

Detection of EP2 and EP3 receptors and 5- and 12-LOX mRNAs by RT-PCR

RNA was isolated from MIN6 cells and mouse islets and reverse-transcribed into cDNA as described previously [30]. MIN6 cell cDNAs were amplified using synthetic oligonucleotide primers designed to amplify 536bp and 380bp products specific for mouse EP2 and EP3 PGE₂ receptors respectively (EP2, sense primer: 5'-GTG GCC CTG GCT CCC GAA AGT C-3'; antisense primer: 5'-GGC AAG GAG CAT ATG GCG AAG GTG-3'; EP3, sense primer: 5'-TGA CCT TTG CCT GCA ACC-3'; antisense primer: 5'-AGC TGG AAG CAT AGT TGG TG-3'). MIN6 cell and mouse islet cDNAs were amplified using primers designed to amplify 128bp and 462bp products specific for mouse 5- and 12-LOX enzymes respectively (5-LOX, sense primer: 5'-CGA CGA CTA CAT CTA CCT CAG C-3'; antisense primer: 5'-CCT CAT CCA CGG TGA CAT C-3'; 12-LOX, sense primer: 5'-TGA TGA CTT GGC TGA GCG AGG ACT-3'; antisense primer: 5'-CTG CCC AGG AGC CAA ACG ACA TT-3'). The amplified PCR products were separated on 1.8% agarose gels, excised using the Qiagen Gel Extraction kit according to the manufacturer's protocol, and their identities were confirmed by sequencing using fluorescent di-deoxy termination methods (Molecular Biology Unit, King's College London).

Statistical Analyses

All numerical data are expressed as means $\pm$ SEM of multiple experiments. Student's *t* tests were used to compare two groups and ANOVA was used for multiple comparisons followed by Bonferroni's test, as appropriate. Values of $p<0.05$ were considered statistically significant.

Fig. 2. Effects of inhibition of cPLA₂ activity on MIN6 cell apoptosis: MIN6 cells were exposed to 2.5mM and 17mM glucose for 48 hours in the absence or presence of MAFP, a cPLA₂ inhibitor, and apoptosis was assessed by measuring caspase-3 activity. 10μM MAFP significantly ($P<0.01$) increased apoptosis at 17mM glucose and 20μM MAFP induced a significant ($P<0.01$) increase in apoptosis at both 2.5mM and 17mM glucose. Data are means±SEM, $n=3-4$.

Results

Effects of overexpressing and/or pharmacological blockade of cPLA₂ on MIN6 cell apoptosis

Exposure of MIN6 cells to exogenous AA (3.1–12.5μM) in the presence of 2.5mM glucose for 48 hours produced a concentration-dependent decrease in caspase-3 activity (Figure 1), suggesting that AA has an anti-apoptotic role in β-cells. In the same experiments, increasing the glucose concentration from 2.5mM to 17mM resulted in a 43% reduction in caspase-3 activity ($P<0.05$), consistent with our previously published data [18] and the low levels of apoptosis at 17mM glucose were not further reduced by the presence of AA (Figure 1). The role of AA in β-cell apoptosis was further investigated by overexpressing cPLA₂ and by inhibiting AA generation. Overexpressing cPLA₂ and thus increasing endogenous AA production in MIN6 cells [28] induced a significant ($P<0.05$) reduction in apoptosis compared to control passage-matched MIN6 cells (caspase activity, OD₄₀₅: non-transfected cells: 0.31 ± 0.03 ; cPLA₂-overexpressing cells: 0.22 ± 0.009 ; mean±SEM, $n=4$, $P<0.05$). These data indicate that both endogenous and exogenous AA protects against apoptosis in MIN6 β-cells.

The role of AA in MIN6 cell apoptosis was also investigated by inhibiting its generation with the cPLA₂ inhibitor MAFP. It can be seen from Figure 2 that exposure of MIN6 cells for 48 hours to 10μM MAFP led to a significant increase in apoptosis at 17mM glucose, but there was no further increase in apoptosis at 2.5mM glucose. However, 20μM MAFP induced an increase in

caspase-3 activity at both 2.5mM and 17mM glucose, and blockade of cPLA₂ activity prevented 17mM glucose from reducing β-cell apoptosis (2.5mM glucose + 20μM MAFP vs 17mM glucose + 20 μM MAFP, $P>0.2$). These results, in which inhibition of AA production induced apoptosis, are consistent with AA exerting a beneficial role on β-cell survival.

Effects of glucose on COX2 mRNA expression in MIN6 cells

AA is metabolised to PGs by COX enzymes, and our previous studies have indicated that COX2 is up-regulated by glucose in human islets [15]. MIN6 cells were incubated for 6, 24 and 48 hours with 2.5mM or 25mM glucose to characterise the time- and glucose-dependent expression of COX2 in this β-cell line. As shown in Figure 3, measurement of COX2 mRNA levels by real-time PCR indicated that MIN6 cells showed stable COX2 mRNA expression at 2.5mM glucose after incubation for 6–48 hours. However, exposure of MIN6 cells to 25mM glucose induced significant increases in COX2 mRNA levels at all time points examined.

Effects of COX2 inhibition on apoptosis in MIN6 cells

We have previously reported that maintenance of MIN6 cells in the presence of glucose (2.5–25mM) resulted in a concentration-dependent decrease in caspase-3 activity [18], over an incubation period (48 hours) at which 25mM glucose induced a maximum up-regulation of COX2 mRNA levels (Figure 3). We therefore investigated the role of COX2 in MIN6 cell apoptosis using a

Fig. 3. Time-dependent effects of glucose on MIN6 cell COX2 mRNA expression: MIN6 cells were exposed to 2.5mM and 25mM glucose for 6, 24 and 48 hours and COX2 mRNA levels were quantified. COX2 mRNA levels were relatively stable at 2.5mM glucose, but exposure of MIN6 cells to 25mM glucose significantly ($P<0.001$) increased COX2 mRNA levels at all time points. Data are means+SEM, $n=3$.

Fig. 4. Effects of COX2 inhibition on MIN6 cell apoptosis: MIN6 cells were exposed to a range of glucose concentrations (2.5, 11, 17 and 25mM) for 48 hours in the absence or presence of a COX2 inhibitor (10 μ M NS-398) and apoptosis was assessed by measuring caspase-3 activity. Inhibition of COX2 caused a significant ($P<0.05$) increase in apoptosis at all glucose concentrations. Data are means+SEM, $n=3$.

selective COX2 inhibitor, NS-398. It can be seen from Figure 4 that glucose inhibited apoptosis, with maximal effects at 17-25mM glucose, and that inhibition of COX2 with 10 μ M NS-398 for 48 hours induced an increase in caspase-3 activity (22-32%) at all glucose concentrations.

Higher concentrations of NS-398 also increased MIN6 cell caspase-3 activity at both 2.5mM (NS-398, 50 μ M: 35% increase; 100 μ M: 220% increase) and 17mM glucose (NS-398, 50 μ M: 76% increase; 100 μ M: 70% increase), but glucose still reduced MIN6 cell apoptosis even in the presence of complete COX2 inhibition (caspase-3 activity, OD₄₀₅, 2.5mM glucose: 0.12 $\pm$ 0.005, + 100 μ M NS-398: 0.29 $\pm$ 0.025, $n=4$, $P<0.001$; 17mM glucose: 0.05 $\pm$ 0.004, + 100 μ M NS-398: 0.09 $\pm$ 0.009;

$n=4$, $P<0.05$). These results indicate that selective inhibition of COX2 at 10, 50 and 100 μ M NS-398 increased apoptosis in MIN6 cells, irrespective of the prevailing glucose concentration, implying that COX2 has an anti-apoptotic role in β -cells but that COX2-generated products are not essential for the anti-apoptotic effects of glucose.

Effects of prostaglandin E₂ on MIN6 cell apoptosis

The most likely explanation for the increase in apoptosis seen after COX2 inhibition with NS-398 was a reduction in PGE₂ levels. To determine whether PGE₂ could confer protection against apoptosis in MIN6 cells, as it has been reported to do in RIN insulinoma cells [23],

Fig. 5. *A:* Effects of PGE₂ on NS-398-induced MIN6 cell apoptosis: MIN6 cells were exposed to 50μM NS-398 in the presence or absence of exogenous PGE₂ for 48 hours and apoptosis was assessed by measuring caspase-3 activity. 50μM NS-398 significantly ($P<0.05$) increased apoptosis by 77% at 2.5mM glucose. PGE₂ (0.2–5μM) caused a concentration-dependent decrease in NS-398-induced apoptosis, with maximum reduction at 5μM PGE₂ ($P>0.2$ versus 2.5mM glucose). Data are means±SEM, $n=3$. *B:* Effects of PGE₂ on MIN6 cell apoptosis: MIN6 cells were exposed to a range of glucose concentrations (2.5, 11, 25mM) in the absence or presence of 1μM PGE₂ for 48 hours and apoptosis was assessed by measuring caspase-3 activity. Exogenous PGE₂ caused a significant ($P<0.05$) reduction in apoptosis at both 2.5mM and 11mM glucose, but not at 25mM glucose. Data are means±SEM, $n=3$. *C:* RT-PCR analysis of EP2 and EP3 receptor mRNA expression in MIN6 cells: MIN6 cell cDNAs were amplified on a Px2 thermocycler with mouse EP2 and EP3 PGE₂ receptor-specific primers over temperature gradients of 61–63°C for EP2 and 58–60°C for EP3. The 1.8% agarose gels confirmed the appropriate sizes (536 bp and 380 bp) of the PCR products and DNA sequencing indicated 99.9% sequence homology between amplified products and predicted nucleotide sequences. M = size marker, HpaII digest of pBluescript SK⁺.

MIN6 cells were maintained in culture for 48 hours with 50μM NS-398 in the absence or presence of PGE₂ and then caspase-3 activity levels were measured. It can be seen from Figure 5a that NS-398 increased caspase-3 activity at 2.5mM glucose and concurrent exposure to PGE₂ (0.2–5μM) rescued the NS-398-induced rise in apoptosis in a concentration-dependent manner, such that caspase-3 activity in the presence of 50μM NS-398 and 5μM PGE₂ was not significantly higher than that at 2.5mM glucose alone ($P>0.2$). Consistent with these data, exogenous PGE₂ (1μM) reduced caspase-3 activity by approximately 20% at both low (2.5mM) and intermediate (11mM) glucose concentrations but not at 25mM glucose (Figure 5b) where rates of apoptosis were already very low. RT-PCR analyses over 3°C annealing temperature gradients indicated that MIN6 cells express mRNAs encoding EP2 and EP3 PGE₂ receptors (Figure 5c) with amplification of products of the appropriate sizes (536bp and 380bp respectively) at all annealing temperatures used.

Effects of inhibition of 5- and 12- lipoxygenase enzymes on MIN6 cell apoptosis

As well as being a substrate for PG synthesis by COX enzymes, AA may also be metabolised to leukotrienes and HETES by lipoxygenase (LOX) enzymes. We have recently identified 5- and 12-LOX in human islets [31] and it can be seen from Figure 6 that 5-

Fig. 6. RT-PCR analysis of 5- and 12-LOX mRNA expression in MIN6 cells and mouse islets: MIN6 cell (MIN6) and mouse islet (islet) cDNAs were amplified on a Px2 thermocycler at 59°C with mouse 5- and 12-LOX-specific primers. The 1.8% agarose gels confirmed the appropriate sizes (128 bp and 462 bp) of the PCR products for the 5- (left panel) and 12-LOX (right panel) enzymes in both MIN6 cells and mouse islets. M = size marker, HpaII digest of pBluescript SK⁺.

The role of LOX enzymes in MIN6 cell apoptosis was investigated using REV5901, a 5-LOX inhibitor, and cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC) and baicalein, selective inhibitors of 12-LOX. Exposure of MIN6 cells for 48 hours to REV5901 (0.25–2 μ M), baicalein (0.25–2 μ M) or CDC (0.0625–1.5 μ M) did not significantly alter MIN6 cell caspase-3 activity at either 2.5mM or 17mM glucose (Table 1), indicating that AA metabolism through the LOX pathways is not a key regulator of apoptosis in MIN6 β -cells.

Effects of AA and PGE₂ on mouse islet apoptosis

MIN6 β -cells were used for many of the experiments described in this study as they are readily available in the large quantities required for reproducible measurements of caspase-3 activity and they allow measurements of apoptosis in β -cells rather than the mixed cell populations of primary mouse islets in which approximately 30% of the islet cell mass is composed of non β -cells. However, cell signalling events in insulin-secreting cells do not always reflect those that occur in primary islets, so we also measured the effects of AA and PGE₂ on caspase-3 activity in isolated mouse islets. Levels of apoptosis in mouse islets that had been maintained at 2.5mM glucose in the absence of serum for 48 hours were low, with a minimum of 50 μ g islet protein being required for detectable measurements of caspase-3 activity. Under circumstances where caspase-3 activity was readily detectable at 2.5mM glucose exposure of islets to 12.5 μ M AA or 1 μ M PGE₂ caused significant reductions in apoptosis (Figure 7), confirming the observations made in the MIN6 β -cell line.

Treatment	Caspase-3 activity	
	2.5mM glucose (% 2.5mM glucose)	17mM glucose (% 17mM glucose)
2.5 or 17mM glucose	100 $\pm$ 2.2	100 $\pm$ 3.9
0.25 μ M baicalein	97 $\pm$ 5	99 $\pm$ 2.6
0.5 μ M baicalein	96 $\pm$ 4.3	102 $\pm$ 5.5
1 μ M baicalein	106 $\pm$ 5.4	98 $\pm$ 4.4
2 μ M baicalein	111 $\pm$ 4.7	93 $\pm$ 2.7
0.25 μ M REV5901	90.9 $\pm$ 4.9	98 $\pm$ 3.2
0.5 μ M REV5901	85.6 $\pm$ 6.2	100.5 $\pm$ 6.7
1 μ M REV5901	87.5 $\pm$ 6.3	105.4 $\pm$ 3.4
2 μ M REV5901	97.1 $\pm$ 4.8	103.3 $\pm$ 2.9
0.0625 μ M CDC	91.5 $\pm$ 5.5	-
0.125 μ M CDC	98.9 $\pm$ 6.3	-
0.25 μ M CDC	114.3 $\pm$ 10.5	-
0.5 μ M CDC	95 $\pm$ 5.7	-
1 μ M CDC	113.7 $\pm$ 4.9	-
1.5 μ M CDC	97.7 $\pm$ 5.5	-

Table 1. Effect of 5-LOX and 12-LOX inhibition on MIN6 cell apoptosis: Inhibition of 12-LOX with 0.25–2 μ M baicalein or 0.0625–1.5 μ M CDC, or 5-LOX with 0.25–2 μ M REV5901 did not significantly affect the rate of MIN6 cell apoptosis. Data are means $\pm$ SEM, n=3 for baicalein and CDC and n=4 for REV5901.

Fig. 7. Effects of AA and PGE₂ on mouse islet caspase 3 activity: Isolated mouse islets were maintained in culture for 40 hours at 2.5mM glucose in the absence or presence of 12.5 μ M AA or 1 μ M PGE₂ and apoptosis was assessed by measuring caspase-3 activity. Both AA and PGE₂ caused a significant (P<0.01) decrease in apoptosis. Data are means $\pm$ SEM; n=3.

Discussion

Glucose increases islet cPLA₂ activity and AA release [12, 13, 32] and up-regulates COX2 in human islets [15, 16], and it also protects MIN6 β -cells from undergoing apoptosis [18]. AA and PGE₂, the major PG synthe-

sised by β -cells, are reported to protect against apoptosis in RIN insulin-secreting cells [22, 23] and to prevent alloxan-induced diabetes in vivo [24, 25], so in the current study we examined whether glucose-stimulated elevations in AA and up-regulation of COX2 play a causal role in the anti-apoptotic effects of glucose.

The roles played by prostaglandins in reducing apoptosis in numerous cell types are fairly well-established. Thus, in addition to exerting anti-apoptotic effects in RIN cells [23], it has been reported that PGE₂ inhibits apoptosis in a variety of cell types including human colon cancer cells [8]], dendritic cells [9, 33], oesophageal adenocarcinoma cells [34], and, most recently, mouse embryonic stem cells [10]. However, cytokines up-regulate COX2 in islets [35] and RINm5F cells [36] and it has been proposed that increased COX2 expression may contribute to islet dysfunction in diabetes [16]. Consistent with a potentially deleterious role for PGE₂ in islets, a 2.5-fold increase in pancreatic PGE₂ levels in transgenic mice overexpressing COX2 and PGE synthase-1 in β -cells was associated with reduced proliferation and decreased β -cell number [37]. However, no apoptotic islet cells could be identified in pancreatic sections from mice in which PGE₂ had been up-regulated [37], so the reduction in β -cell mass was not related to increased apoptosis.

In contrast to the protective role ascribed to AA in RIN cells [22] a number of published studies suggest that AA exerts deleterious effects on cells by promoting apoptosis [3-5]. It is noticeable from the published data that the pro-apoptotic effects of AA are observed when it is used at high concentrations (=100 μ M), but it has been estimated that exposure of islets to stimulatory concentrations of glucose causes AA levels to reach only approximately 35 μ M [38]. Consistent with this, AA protected against alloxan-induced cytotoxicity in RIN cells when used at 15 μ M [22], and AA was reported to suppress apoptosis in W256 carcinoma cells with maximal effects at 25 μ M [6]. In addition, studies in human granulosa cells indicated that AA protected against palmitate-induced apoptosis over the range 3-15 μ M, but enhanced the pro-apoptotic effects of palmitate at concentrations =30 μ M [2]. Given the potential pro-apoptotic effects of AA at supraphysiological concentrations it was used here over the range 3.1-12.5 μ M.

In the current study AA decreased caspase-3 activity in MIN6 β -cells in a concentration-dependent manner, and a similar protective effect of AA was observed in mouse islets. We also found that glucose elevated COX2 mRNA levels in MIN6 cells, as it did in human islets [15, 16]. The protective effects of AA are most likely through

its metabolism to PGE₂ since selective inhibition of the COX2 pathway with NS-398 enhanced apoptosis in MIN6 cells and this was rescued by providing exogenous PGE₂ to circumvent the COX2 blockade. The increase in apoptosis after COX2 inhibition in MIN6 β -cells is in agreement with many earlier studies in which COX2-specific and non specific non-steroidal anti-inflammatory drugs caused apoptosis (reviewed in [39]). In a similar manner, it has been found that overexpression of COX2 protected against apoptosis in intestinal epithelial cells and this effect could be reversed by COX inhibition [7]. Our observations of PGE₂-mediated protection in MIN6 cells is also consistent with earlier reports in which PGE₂ inhibited COX2 inhibitor-induced apoptosis in a variety of cell types [8, 40] and in which exogenous PGE₂ alone protected against apoptosis [9, 10, 19, 20]. Taken together, these data are consistent with AA metabolism through the COX2 pathway generating PGE₂, the major β -cell prostaglandin, to reduce the entry of cells into the programmed death pathway. However, our observations that 17mM glucose still protected MIN6 cells against apoptosis even under conditions where PGE₂ synthesis would be fully inhibited in the presence of 100 μ M NS398 indicate that glucose-stimulated COX2 upregulation is not the major mechanism by which glucose exerts its anti-apoptotic effects in MIN6 β -cells.

Defining the mechanisms by which PGE₂ decreases β -cell apoptosis is beyond the scope of the current study. However, experiments in other cell types have indicated that PGE₂ can exert autocrine or paracrine effects at cell surface EP receptors to stimulate a variety of signalling pathways including cyclic AMP generation [19, 20]; expression of the anti-apoptotic protein Bcl-2 [19, 41]; PI-3-kinase/PKB activation [9, 33] and JNK activation [33]. The protective effects of PGE₂ have been reported to depend either on expression of EP2 receptors [10, 19] or EP4 receptors [34] or on both EP2 and EP4 receptors [9, 42, 42]. In the current study we have identified mRNAs encoding EP2 and EP3 PGE₂ receptors in MIN6 cells. EP2 receptors are linked to cyclic AMP generation [43], and it has been proposed that PGE₂ protects RIN insulin-secreting cells against alloxan-induced apoptosis through its ability to increase cyclic AMP [23], so it is possible that PGE₂ acts through a similar mechanism in MIN6 cells and mouse islets.

In addition to COX-products of AA metabolism having anti-apoptotic potential, it has been reported that inhibition of 5-LOX induces apoptosis and that 5-HETE and LTB₄, 5-LOX metabolites, inhibit apoptosis [44-46]. Similarly, 12-LOX inhibitors are reported to increase apoptosis

[42, 47] as a consequence of decreased 12-HETE generation, and addition of 12-HETE in the presence or absence of 12-LOX inhibition reduced apoptosis [48, 49]. Given the possible protective effects of AA metabolism through LOX pathways we therefore also investigated whether inhibition of LOX enzymes affected MIN6 cell apoptosis, as assessed by caspase-3 activity. RT-PCR analyses indicated that MIN6 cells and mouse islets expressed mRNAs for 5- and 12-LOX, as we have previously reported for human islets [31]. However, in contrast to the data obtained using NS-398, inhibition of 5-LOX and 12-LOX did not affect MIN6 cell caspase-3 activity. These observations support the conclusion that the increase in apoptosis observed with NS-398 results from inhibition of anti-apoptotic prostaglandin production and not to the generation of potentially pro-apoptotic LOX products (leukotrienes, HETEs) due to an increase in AA availability after COX2 inhibition.

In summary, we have demonstrated that AA has an anti-apoptotic role in the MIN6 mouse β -cell line and mouse islets, and that this protective effect is most likely through its metabolism to PGE₂ via the COX2 pathway. The anti-apoptotic role of PGE₂ in β -cells could be important to islet physiology in diabetes. Thus, although COX2 has been proposed to play a role in islet dysfunction

[16] the inability of COX2 inhibitors to prevent cytokine-mediated islet destruction and the absence of β -cell damage in response to exogenous PGE₂ [50] suggest a dissociation between cytokine-induced COX2 upregulation [35;36] and β -cell destruction. Furthermore, the upregulation of COX2 in β -cells in the NOD mouse model of Type 1 diabetes [51] could be a protective response to the diabetic environment rather than a causal link in β -cell destruction. Our data suggest that the PGE₂ generated under these circumstances would protect the β -cells from cytokine-mediated apoptosis. Consistent with this, the surviving β -cells in islets from NOD mice showing advanced insulinitis expressed both COX2 and insulin [51]. In addition, the up-regulation of both AA and PGE₂ that occurs under hyperglycaemic conditions [12-14] may act as a defence mechanism to protect β -cells under conditions of enhanced apoptosis such as those experienced in response to elevated saturated fatty acids, which are known to predispose towards Type 2 diabetes.

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