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Cytochrome P450 1A-like proteins expressed in the islets of Langerhans and altered pancreatic β-cell secretory responsiveness

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1 The cytochrome P450 (CYP) mixed-function oxidase system is widely distributed in body tissues and plays a key role in the metabolism of endogenous and exogenous compounds. Little attention has been paid to the expression of the system in the islets of Langerhans. The current study has examined the expression and potential role of the CYP1A family within the islets of Langerhans of control and 3-methylcholanthrene (3-MC)-induced Wistar rats.

2 CYP1A expression within pancreatic slices and islets from 3-MC-induced and control rats demonstrated that CYP1A-like protein levels were induced by 3-MC pretreatment (25 mg kg^{-1} day^{-1}; i.p. for 3 days).

3 Effects of 3-MC-induction on β-cell secretory responsiveness were investigated by use of rat collagenase-isolated islets. Insulin release from control islets incubated with 3 mM glucose (basal) was 1.4 ± 0.2 ng/islet h^{-1} (mean ± s.e.mean, $n=7$). Incubation with 16.7 mM glucose, 25 mM KCl, 100 μM arachidonic acid, or 100 μM carbachol caused a 4.4, 7.0, 4.0 and 4.2 fold, respectively, increase in insulin release ($P<0.001$). Forskolin (2 μM), or phorbol 12-myristic 13-acetate (10 nM) potentiated glucose-stimulated insulin release 1.2 and 1.6 fold ($P<0.01$) whereas adenalin (1 μM) caused a 76% inhibition ($P<0.01$).

4 Islets from 3-MC pretreated animals displayed similar responsiveness to all agents tested except arachidonic acid, carbachol and forskolin. Insulin release in response to arachidonic acid and carbachol was enhanced 5.2 and 5.0 fold, respectively, by 3-MC pretreatment ($P<0.001$ compared to control islets incubated with 3 mM glucose); the effect of forskolin on insulin output was significantly decreased (20%; $P<0.01$) compared to control islets.

5 3-MC pretreatment did not cause any significant differences in food intake, plasma glucose or total islet insulin content. Incubation of islets with 3-MC *in vitro* (1 μM – 10 mM) did not affect basal or glucose-stimulated insulin release.

6 These data suggest that CYP1A-like protein expression within the pancreatic islets of Langerhans is inducible and may have a role in the alteration of pancreatic β-cell secretory responsiveness.

Keywords: Cytochrome P450; CYP1A; pancreatic β-cell; diabetes; insulin secretion

Introduction

The most important enzyme system involved with xenobiotic metabolism is the cytochrome P450-dependent mixed function oxidase system, being expressed in almost every tissue (Gonzalez, 1989). It functions as an electron transport chain and requires nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. Two enzymes are involved in the transport chain, the flavin-containing cytochrome P450-reductase and the haemoprotein cytochrome P450. The latter acts as the terminal oxygenase and confers the substrate specificity to the system. Cytochrome P450 exists as a number of families, each comprising one or more proteins, which differ from each other in their substrate specificity (Gonzalez, 1989). The cytochrome P450 isoforms primarily involved in xenobiotic metabolism are highly inducible by chemical exposure and pathological conditions such as diabetes mellitus (Barnett *et al*., 1990a,c). Insulin-dependent diabetes mellitus causes profound alterations in hepatic cytochrome P450 expression of toxicological importance for xenobiotic metabolism (Barnett *et al*., 1990b).

The cytochrome P450 system has been shown to be expressed within the pancreas (Foster *et al*., 1993). The pancreatic β-cells, within the islets of Langerhans, are highly metabolically active and richly vascularized, being exposed to many xenobiotics in the blood supply. As the cytochrome P450 (CYP) system is involved in the bioactivation of numerous xenobiotics, expression of this system within the pancreatic β-cell may facilitate reactive intermediate production. The CYP1A family, in particular, is associated with the bioactivation of numerous environmental chemicals, drugs and chemical carcinogens of planar or near planar structure. The precise role of CYP1A proteins in endogenous metabolism has not been clearly defined, although they have been shown to metabolize arachidonic acid *in vitro* and are expressed in most tissues including the pancreas (Falck *et al*., 1983; Foster *et al*., 1993).

Xenobiotic-induced alteration of cytochrome P450 expression in the islets of Langerhans may be of functional and toxicological significance. In particular, pancreatic β-cells are very susceptible to xenobiotic-mediated damage (Dunn *et al*., 1943; Rakieten *et al*., 1963; Dulin & Soret, 1977). This has been suggested to be a consequence of low levels of protective enzymes (Malaisse, 1982). Therefore, the current investigation has examined the expression and inducibility of the CYP1A family in the rat islets of Langerhans and the consequences of this expression with respect to pancreatic β-cell insulin secretory responsiveness.

Methods

Animals

Male Wistar albino rats (250–350 g) were obtained from the Biomedical and Behavioural Research Unit, University of

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Ulster. Animals were supplied with a standard pellet diet and water *ad libitum*. Induction of CYP1A proteins was performed by treating the rats ($n=40$) with 3-methylcholanthrene (3-MC) in corn oil at 25 mg kg^{-1} body weight for three days by intraperitoneal injection. Control animals ($n=40$) received intraperitoneal injection of corn oil only for three days. All animals were killed 24 h following the final injection and terminal blood samples obtained. Liver and pancreas were excised from each animal and used for the following investigations.

Immunocytochemistry

Samples of liver and pancreas from groups of control and 3-MC-treated rats were excised and fixed in 4% paraformaldehyde overnight. This was then replaced with 5% w/v sucrose and after a further 24 h replaced with 30% w/v sucrose/1% w/v sodium azide/69% water for 24 h. The tissues were sectioned at 10–12 μm at −20°C with a cryomicrotome and mounted onto subbed slides (gelatin and chrome alum). The slides were air dried at room temperature for 30 min. The slides were washed in phosphate buffered saline (PBS), pH 7.4 for 20 min and then incubated at 4°C for 24 h with polyclonal antibody to CYP1A (1 : 400 dilution). Unbound antibody was removed by washing the slides in PBS (20 min). The bound antibody was visualized by applying secondary antiserum rabbit anti-sheep fluroisothiocyanate linked (RAS-FITC), and incubating (in the dark) for 30 min. Unbound antibody was again removed by washing in PBS for 20 min. Sections were mounted in PBS/glycerol/antifade (Antifade; Molecular Probes Europe, Leiden, Netherlands) and examined with a fluorescent image analysis microscope (Nikon, U.K.), and photographed with a polaroid quick print video printer (Polaroid, U.K.).

Islets of Langerhans were isolated from rat pancreata by collagenase digestion by a modification of the method of Lacy & Kostianovsky (1967). A collagenase-containing (10 ml, 1 mg ml^{-1}), Hanks buffered saline solution (HBSS; comprising (mM): KCl 5.4, KH_2PO_4 0.44, NaCl 137, Na_2HPO_4 0.63 and D-glucose 5.6; supplemented with 10 mM HEPES and 5% v/v foetal calf serum, pH 7.4), was used to distend the pancreas *in situ*, by injection into the common bile duct. The distended pancreas was removed and incubated at 37°C for 40 min. Ten millilitres of collagenase-free HBSS was then added and the pancreas shaken vigorously for 20 s to facilitate separation of intact islets from digested acinar tissue. The pancreatic digest was washed twice with HBSS (10 ml) and intact islets were selected by use of a finely drawn glass micro-pipette under a binocular microscope.

Western blotting

Microsomal fractions of liver and isolated islets of Langerhans were prepared according to the method of Ioannides & Parke (1975). Total microsomal protein was determined with a Bio-Rad protein kit (Richmond, CA, U.S.A.) by use of a standard curve constructed from a range of known bovine serum albumin standards from 2 μg ml^{-1} to 40 μg ml^{-1}. Microsomal liver (40 μg) or islet (100 μg) protein samples were separated by SDS/polyacrylamide-gel electrophoresis by a modification of the method of Laemmli (1970). Following separation, the microsomal proteins were transferred to nitrocellulose membrane (Towbin *et al*., 1979). The nitrocellulose membrane was then probed for CYP1A proteins by use of the polyclonal antibody to CYP1A (1 : 10 000 dilution) and visualized with an alkaline-phosphatase-linked secondary antibody and 5-bromo-4-chloro-3-indophosphate/nitroblue tetrazolium (BCIP/NBT) as substrate.

Insulin secretory responsiveness and islet insulin content

For insulin secretion studies, freshly isolated islets were pre-incubated for 60 min at 37°C in 500 μl Krebs-Ringer Bicarbonate Buffer (KRB; composition in mM: NaCl 115, KCl 4.7, $CaCl_2.6H_2O$ 1.28, $MgSO_4$ 1.2 and Na_2HCO_3 10; pH 7.4) supplemented with 3 mM D-glucose.

Five groups of 3 islets were subsequently incubated for 60 min in 1 ml KRB supplemented with 3 or 16.7 mM D-glucose and established modulators of insulin secretion indicated in Table 1. At the end of the incubation period aliquots of the buffer were removed and stored at −20°C for subsequent insulin assay. Determination of total islet insulin was performed by sonication (3 cycles, 10 s duration in KRB) and overnight extraction of groups of 3 islets ($n=5$) in 500 μl ice-cold acid-ethanol (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) water), at 4°C. Islets were then sonicated a second time and aliquots of centrifuged supernatant were stored at −20°C for subsequent determination of insulin content.

Chemicals

Collagenase (P) was purchased from Boeringer Mannheim, U.K. Bovine serum albumin (BSA Fraction V), arachidonic acid, adrenaline, 3-methylcholanthrene, 5-bromo-4-chloro-3-indophosphate/nitroblue tetrazolium (BCIP/NBT), co-factors and general reagents were purchased from the Sigma Chemical Co (Poole, Dorset, U.K.). The polyclonal CYP1A antibody was kindly provided by Dr Costas Ioannides (University of Surrey). This antibody was raised in sheep against rat CYP1A proteins and recognises both CYP1A1 and CYP1A2 proteins in rat liver microsomal preparations following SDS-PAGE separation and Western blot anaylsis (Barnett *et al*., 1990a).

Analysis

Insulin was measured by dextran charcoal radioimmunoassay (Flatt & Bailey, 1981), by use of guinea-pig anti-porcine insulin antiserum, crystalline rat insulin standard (Novo Industria, Copenhagen, Denmark) and [^{125}I]-bovine insulin (Amersham International, U.K.). Insulin concentration was determined by means of the standard curve constructed from the known standards by use of a spline curve fitting alogarithm. Plasma glucose was determined with a Beckman glucose analyser (Beckman Riic, (TD). High Wycombe, U.K.) based on the glucose oxidase method of Stevens (1971).

Data are presented as mean ± s.e.mean. Statistical analysis was performed by unpaired Student's t test. Differences were considered statistically significant if $P<0.05$.

Results

Immunocytochemical studies of CYP1A proteins in the liver and pancreatic islets of Langerhans demonstrated that CYP1A-like proteins were present in both the liver and pancreatic islets of Langerhans of control rats (Figure 1). The pattern of staining in the islets was roughly even throughout, suggesting expression of CYP1A-like proteins in the islet B-, A-, and D-cells. Following 3-MC pretreatment, the staining of CYP1A proteins appeared to be increased in both tissues following visual analysis. The presence of CYP1A-like proteins and induction by 3-MC pretreatment were confirmed by immunoblotting of the apoprotein levels in the liver and islets with a polyclonal antibody to CYP1A proteins (Figure 2). Both CYP1A1 and CYP1A2 proteins were identified in the 3-MC-induced liver (Figure 2) while only CYP1A1-like protein could be identified in the islet microsomal preparations.

Pretreatment of animals with 3-MC did not result in any significant alteration of plasma glucose (control 8.8 ± 0.3 mM, 3-MC 9.2 ± 0.6 mM; $n=40$) or total insulin content of the islets (control 82.0 ± 1.2 ng/islet, 3-MC 80.7 ± 2.4 ng/islet; $n=5$). Furthermore, preincubation of control islets in KRB with a range of concentrations of 3-MC (1 μM, 10 μM, 100 μM, 1 mM or 10 mM) at non-stimulating (3 mM) or stimulating (16.7 mM) concentrations of glucose did not significantly alter insulin secretion (1.2 ± 0.1 ng/islet h^{-1} at 3 mmol l^{-1} glucose and

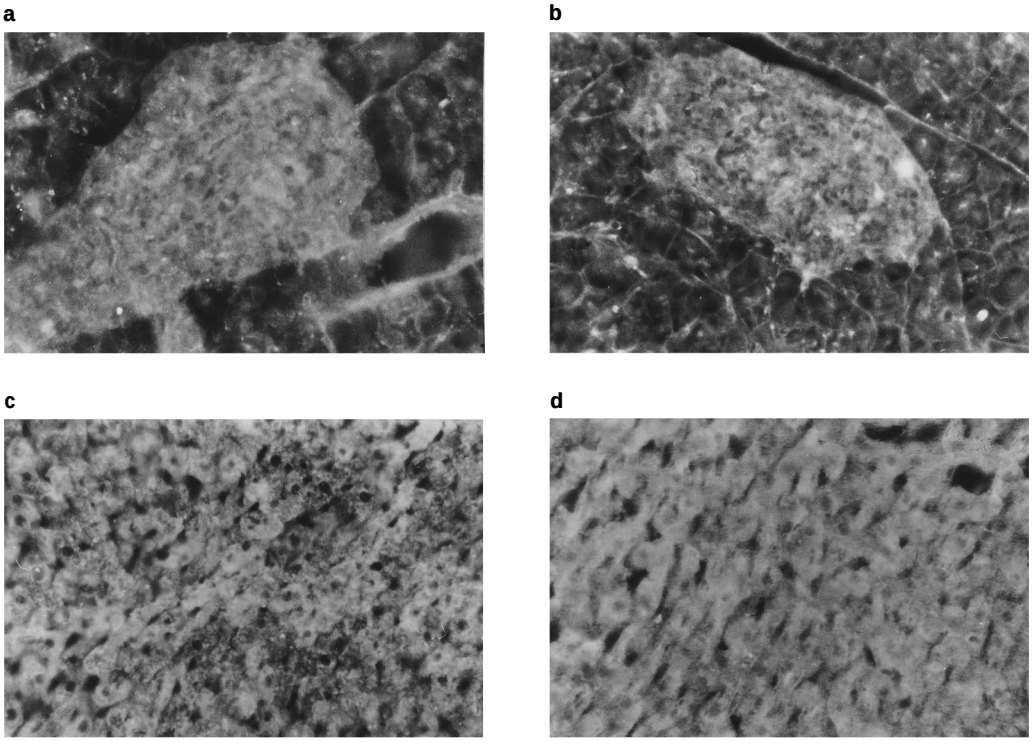

Figure 1 Immunocytochemical localization of CYP1A-like protein expression in liver and pancreatic islets of Langerhans of control and 3-methylchloranthrene (3-MC)-treated animals. Sections were treated with a polyclonal antibody to CYP1A proteins and visualized by a RAS-FITC linked secondary antibody. Control animals: islet (a); liver (c); 3-MC-induced animals: islet (b); liver

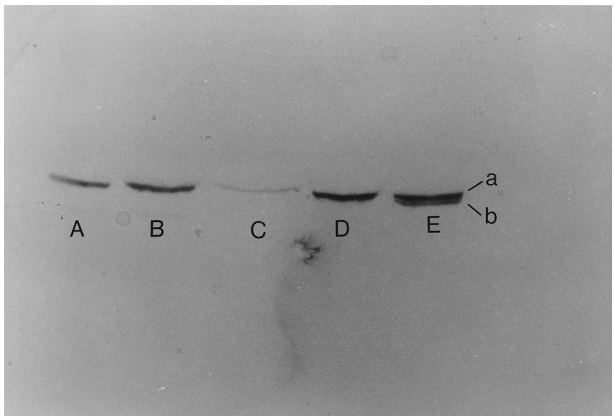

Figure 2 Western blotting of microsomal proteins from liver and islets of Langerhans of control and 3-methylcholanthrene (3-MC)-treated animals. Liver microsomes (40 μg) or islet microsomes (100 μg) were separated by SDS–PAGE and transferred to nitrocellulose. CYP1A-like protein levels were visualized by use of a polyclonal antibody to CYP1A proteins and an alkaline phosphatase linked secondary antibody. Lane A: control islet; lane B: 3-MC-induced islet; lane C: molecular weight marker (58,000d); lane D: control liver; lane E: 3-MC-induced liver (a: CYP1A1, b: CYP1A2).

6.3 ± 0.1 ng/islet h^{-1} at 16.7 mM glucose, respectively, $n = 7$). Glucose 16.7 mM induced a 5 fold stimulation of insulin secretion ($P < 0.001$) in the presence and absence of 3-MC.

The insulin secretory responsiveness of islets isolated from control versus 3-MC treated rats was determined in response to 16.7 mM glucose and other established secretagogoues. Incubation of islets from control rats with 16.7 mM glucose, 25 mM KCl, 100 μM carbachol or 100 μM arachidonic acid elicited significant 4.4, 7.0, 4.2, and 4.0 fold increases in insulin output compared to islets incubated with 3 mM glucose alone (Table 1). Similarly, when islets from control rats were incubated with a stimulating glucose concentration (16.7 mM) and forskolin (25 μM) or phorbol 12-myristate 13-acetate (PMA; 10 nM) insulin secretion was potentiated 1.2 and 1.7 fold, respectively, when compared to control islets incubated with 16.7 mM glucose alone (Table 1). Furthermore, the stimulant effects of 16.7 mM glucose could be effectively antagonized by the inclusion of 1 μM adrenaline in the incubation medium (Table 1).

Islets derived from animals pretreated with 3-MC demonstrated similar insulin secretory responsiveness to those of the control islets when incubated with 3 mM glucose, 16.7 mM glucose, 25 mM KCl, 10 nM PMA or 1 μM adrenaline (Table 1). Incubation of the islets from 3-MC-treated rats with 25 μM forskolin and 16.7 mM glucose caused significantly less potentiation of insulin output compared to control islets incubated under the same conditions (Table 1). The insulin secretory response to carbachol was significantly greater in islets isolated from 3-MC-treated rats (Table 1).

In comparison to the control islets, islets from 3-MC-treated rats demonstrated a significantly greater insulin response to arachidonic acid (100 μM; Table 1). Investigation of insulin secretory responsiveness to arachidonic acid over a range of concentrations (1–100 μM) demonstrated a significant stepwise enhancement of insulin output from 2 μM upwards (Figure 3) when compared to islets from control rats.

Discussion

The CYP1A family of proteins are expressed in most mammalian tissues (Gonzalez, 1989). Although these proteins have a clearly defined role in the bioactivation of many environmental chemicals the precise endogenous role of the enzymes has not been clearly established. We have demonstrated that CYP1A1-like protein is expressed within the β-cells of the islets of Langerhans and most other islet cells. Furthermore, expression of CYP1A1-like protein can be induced by pretreatment of animals with the inducing agent 3-MC. However,

Table 1 Insulin release from pancreatic islets of Langerhans from control and 3-methylcholanthrene (3-MC)-induced animals in response to various stimulating agents

		Insulin secretion (ng/islet h^{-1})	
Addition	*Glucose* (mM)	*Control animals*	*3-MC animals*
None	3	1.4±0.2	1.5±0.2
None	16.7	6.2±0.3	6.2±0.2
KCl (25 mM)	3	9.8±0.5***	9.9±0.4***
Forskolin (25 μM)	16.7	7.6±0.3**	6.3±0.4†
PMA (10 nM)	16.7	10.2±0.3***	10.0±0.3***
Carbachol (100 μM)	3	6.0±0.3***	7.1±0.4††***
Arachidonic acid (100 μM)	3	5.5±0.3***	7.4±0.3†††***
Adrenaline (1 μM)	16.7	1.5±0.2***	1.6±0.3***

Values are mean±s.e.mean of 7 separate experiments. $^{**}P<0.01$, $^{***}P<0.001$ compared to islets incubated in the same glucose concentration in the absence of test agent. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$ $^{\dagger\dagger\dagger}P<0.001$ compared to control islets incubated in the same test agent.

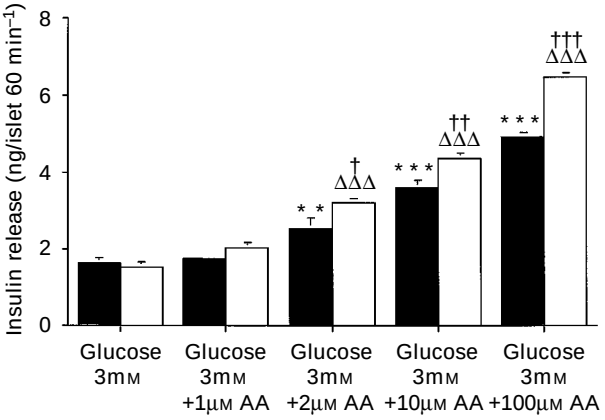

Figure 3 Insulin secretory responsiveness of control and 3-methylcholanthrene (3-MC)-induced islets in response to arachidonic acid. Control and 3-MC-induced islets ($n=7$) were incubated in 3 mM glucose with a range of arachidonic acid (AA) concentrations for 20 min. Insulin secretion was determined by radioimmunoassay. $^{**}P<0.01$, $^{***}P<0.001$ compared to 3 mM glucose for control islets. $^{\Delta\Delta\Delta}P<0.001$ compared to 3 mM glucose for 3-MC-induced islets. $^{\dagger\dagger\dagger}P<0.001$; $^{\dagger\dagger}P<0.01$; $^{\dagger}P<0.05$ compared to control islets. Open columns, 3-MC-induced islets; solid columns, control islets.

whether this protein is CYP1A1 holoprotein and represents part of a fully functional islet cytochrome P450 mixed-function oxidase system, has yet to be established.

Investigation of the effects of induction on the secretory responsiveness of pancreatic β-cells was performed by use of a range of nutrient and non-nutrient secretagogues, selected to examine possible effects on the various pathways of stimulus-secretion coupling within the pancreatic β-cells. 3-MC-pretreatment of animals was not associated with pancreatic islet cell dysfunction *per se* as demonstrated by the lack of effect on plasma glucose, islet insulin content, and both basal and glucose-induced secretion. 3-MC also lacked effects on insulin secretion *in vitro* when tested over a wide concentration range. This suggests that islet glucose metabolism, glucose-induced closure of the adenosine 5′-triphosphate (ATP)-sensitive potassium channels, and the rates of insulin biosynthesis and degradation were unaltered by 3-MC pretreatment. Furthermore, since the stimulant effect of glucose on insulin secretion could be effectively antagonized by adrenaline in islets from both groups of rats, the receptor-mediated inhibition of insulin secretion appears to be unchanged by 3-MC pretreatment.

However, 3-MC-pretreatment was associated with selective effects on pancreatic β-cell function, including a significant reduction in forskolin-induced insulin release. Forskolin promotes insulin secretion by directly activating the catalytic subunit of adenylate cyclase and thereby promotes the formation of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) within the pancreatic β-cell (Ullrich & Wollheim, 1984). The observed effect of 3-MC pretreatment is rather unexpected. However, since forskolin is a plant diterpene with a planar structure it may be a possible substrate for CYP1A-like proteins. If the islets contain a fully functional cytochrome P450 system, it is possible that increased forskolin metabolism during the incubation may have diminished its stimulant effect on adenylate cyclase activity, thereby decreasing insulin secretion from islets of 3-MC-pretreatment animals.

The ability of PMA to stimulate insulin secretion through the activation of protein kinase C (Best & Malaisse, 1983; Dunlop & Larkins, 1986; Peter-Reisch *et al*., 1988) was unaffected by 3-MC-pretreatment. In contrast, carbachol-induced insulin secretion (receptor-mediated activation of phospholipase C resulting in diacylglycerol (DAG) production, activation of PKC and inositol triphosphate-stimulated Ca^{2+} release from endogenous Ca^{2+} stores (Berggren *et al*., 1994) was significantly enhanced by 3-MC-pretreatment. It has been postulated that the cytochrome P450 system is involved in Ca^{2+} mobilization, which may explain the enhanced effect of 3-MC pretreatment on insulin secretion (Alvarez *et al*., 1991). However, arachidonic acid is often encountered as one of the acyl groups liberated by receptor activation of membrane phospholipases. Indeed, carbachol has been shown in islets to increase arachidonic acid levels by the action of diacyl glycerol lipase on DAG (Konrad *et al*., 1992). As such, it is possible that the enhanced effect of carbachol may be mediated via the increased availability of arachidonic acid for metabolism.

Arachidonic acid, a known stimulator of insulin secretion (Wolf *et al*., 1986; 1991; Metz *et al*., 1987; Metz, 1988; Band *et al*., 1992) was significantly more potent at stimulating insulin release in the 3-MC-pretreated islets. Furthermore, a significant stepwise increase in insulin release could be elicited by incubation of both groups of islets with a range of arachidonic acid concentrations (Figure 3). 3-MC-pretreatment was associated with significantly increased insulin secretion above that of control islets from 2–100 μM arachidonic acid (Figure 3).

The role of arachidonic acid as a messenger molecule within a variety of biological systems, including insulin secreting cells, has been clearly established (Neddleman *et al*., 1986; Metz *et al*., 1987; Metz, 1988; McGiff, 1991; Smith *et al*., 1991; Band *et al*., 1992). Arachidonic acid has been shown to stimulate insulin secretion from pancreatic β-cells by a variety of mechanisms, including activation of PKC, mobilization of Ca^{2+} from intracellular stores and metabolism to a plethora of signal molecules (Smith, 1992; Ford-Hutchinson *et al*., 1994; Wolf *et al*., 1994; Capdevila *et al*., 1995). Since the secretory responses of islets to activation of PKC with PMA or to stimulation of Ca^{2+} influx with 25 mM KCl were unchanged by 3-MC-pretreatment, attention is clearly focused on the possible effects of 3-MC-pretreatment on the modulation of ara-

chidonic acid metabolism. Inhibition of both the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism does not result in complete inhibition of arachidonic acid-stimulated insulin release (Basudev *et al*., 1993). Arachidonic acid metabolism by cytochrome P450 isoforms, including CYP1A and CYP4A families, via the epoxygenase pathway produces a range of *cis*-epoxyeicosatrienoic acids (EETs) and derivatives (Capdevila *et al*., 1981; 1983; Morrison & Pascoe, 1981; Oliw & Oates, 1981). These products have been shown to have a variety of biological activities *in vitro* including the ability to simulate insulin secretion from insulin secreting cell lines (Capdevila *et al*., 1995). Although the CYP4A family appears to be the most selective of the cytochrome P450s for arachidonic acid metabolism, the CYP1A family has been shown to metabolize arachidonic acid via the epoxygenase pathway. As such, it is possible that the selective effects of 3-MC pretreatment on insulin secretion may be mediated by islet CYP1A1-like protein metabolism of arachidonic acid to stimulator metabolites. However, further studies are required to elucidate fully the mechanisms responsible for 3-MC-induced alterations of arachidonic acid-stimulated insulin release.

The induction of the CYP1A family in this study employed a specific inducing agent. However, the CYP1A family of proteins are inducible by numerous environmental chemicals including polyaromatic hydrocarbons present in cigarette smoke, vehicle exhausts and certain smoked foods (Ioannides & Parke, 1990). As such, induction of this family in the pancreatic islets of Langerhans by xenobiotic exposure can be envisaged to affect insulin secretion from the pancreatic β-cell with possible implications for the physiology and pathophysiology of insulin secretion.

In conclusion, the current study demonstrates that CYP1A1-like protein is expressed and inducible within the pancreatic islets of Langerhans. Furthermore, induction of this protein is accompanied by selective enhancement of carbachol and arachidonic acid stimulated insulin secretion. The precise mechanism responsible for such an effect has yet to be fully elucidated. However, CYP1A-mediated metabolism of arachidonic acid to metabolites with stimulating effects on insulin exocytosis merits consideration. Further studies are required to characterize fully the expression of cytochrome P450 proteins with pancreatic β-cells and their possible role in the dysregulation of insulin secretion.

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