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Role of CYP2E1 in ketone-stimulated insulin release in pancreatic B-cells

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Abstract

The role of CYP2E1 in ketone-stimulated insulin release was investigated using isolated pancreatic islets of Langerhans and two mammalian insulin secreting pancreatic β-cell lines engineered to stably express human CYP2E1 (designated BRIN BD11h2E1 and INS-1h2E1). Isolated rat pancreatic islets were shown to express the CYP2E1 isoform which was inducible by pretreatment of animals with acetone. The cDNA encoded CYP2E1 was expressed and inducible in the engineered cells as shown by Western blotting. The transfected protein was enzymatically active in the heterologous cells as determined by *p*-nitrophenol hydroxylation rates (0.176 ± 0.08 vs. 0.341 ± 0.08 nmol/min/mg microsomal protein in BRIN BD11 control cells and BRIN BD11h2E1 cells respectively, $P < 0.001$; 0.204 ± 0.03 vs. 0.633 ± 0.102 nmol/min/mg microsomal protein in INS-1 and INS-1h2E1, respectively, $P < 0.001$). Cultivation of CYP2E1 expressing BRIN BD11h2E1 and INS-1h2E1 cells in 40 mM ethanol increased the rate of *p*-nitrophenol hydroxylation (0.968 ± 0.09 nmol/min/mg microsomal protein, $P < 0.001$ and 0.846 ± 0.103 nmol/min/mg microsomal protein, $P < 0.001$, respectively) providing further evidence that the heterologous protein is inducible. Cultivation of control cells with ethanol had no observable effect (0.186 ± 0.05 and 0.195 ± 0.03 in BRIN BD11 and INS-1, respectively). These cell lines also express NADPH-cytochrome P450 reductase protein which was enzymatically active (0.632 ± 0.023 in parental BRIN BD11 vs. 0.657 ± 0.066 without ethanol and 0.824 ± 0.014 nmol/min/mg microsomal protein with ethanol in BRIN BD11h2E1, $P < 0.05$; and 1.568 ± 0.118 in parental INS-1 vs. 1.607 ± 0.093 without ethanol and 1.805 ± 0.066 nmol/min/mg microsomal protein with ethanol in INS-1h2E1, $P < 0.05$) thereby providing a functional cytochrome P450 system. The insulin secretory response of control cell lines and islets was similar to cell lines and islets which had been chemically pretreated, to induce CYP2E1 expression, in response to known nutrient secretagogues. However, insulin output was significantly higher in pretreated islets (1.3-fold, $P < 0.05$) and CYP2E1 expressing cell lines (BRIN BD11h2E1 2.3-fold, $P < 0.001$; INS1-1h2E1 1.6-fold, $P < 0.001$) when stimulated with the ketone 3-hydroxybutyrate than control islets and parental cell lines respectively. Similar acute exposure to acetoacetate enhanced insulin secretion by 1.3-fold ($P < 0.05$) in pretreated islets, 2.6-fold ($P < 0.001$) in ethanol pretreated BRIN BD11h2E1 and 1.4-fold ($P < 0.001$) in ethanol pretreated INS-1h2E1 cells compared to the respective control islets or ethanol pretreated control parental cells. Therefore, these studies highlight a possible role for CYP2E1 in pancreatic cell dysfunction.

Keywords: Cytochrome P450; CYP2E1; Pancreatic β-cell; Diabetes; Insulin secretion; Ketone

1. Introduction

The vast majority of xenobiotics, whether drugs, food additives, or environmental chemicals undergo metabolism following entry into the body. The major enzyme system involved in xenobiotic metabolism is the cytochrome P450 superfamily [1] which is found in highest concentration in the liver where it is associated with the endoplasmic reticulum. However, it is evident that the P450 system is expressed in many extrahepatic tissues including the pancreas where the CYP2E1 isoform is expressed and inducible in rat [2–4]. The cytochrome P450 system is a superfamily of isoforms each with specific but overlapping substrate specificity [5]. As such, the system is endowed with the ability to metabolize a plethora of structurally diverse substrates. The cytochrome P450 isoforms primarily involved in xenobiotic metabolism are highly inducible

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CYP2E1, cytochrome P450 2E1; HBSS, Hank's buffered saline solution; LSD, least significant difference; PMA, phorbol-12-myristate-13-acetate.

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following chemical exposure and by certain pathological conditions, such as diabetes mellitus [6,7]. Insulin-dependent diabetes mellitus causes profound alterations of hepatic cytochrome P450 expression of toxicological importance for xenobiotic metabolism [8]. Xenobiotic metabolism usually results in the production of more polar products thereby facilitating excretion from the body. However, a paradox of cytochrome P450-mediated metabolism is that deactivation may not be the only outcome. Xenobiotic bioactivation, resulting in the generation of reactive intermediates may cause biomolecular damage resulting in cellular dysfunction and destruction.

The pancreatic B-cells, located within the islets of Langerhans, are highly metabolically active, richly vascularised, and exposed to many chemicals in the blood. As the cytochrome P450 system is involved in the metabolism of numerous xenobiotics, expression of this system within the pancreatic B-cell may be of toxicological importance for B-cell dysfunction. In particular, the CYP2E1 isoform, is associated with the bioactivation of numerous chemicals including; paracetamol, nitrosamines, and halogenated alkanes [9]. The expression of this isoform is regulated at the levels of gene transcription [10], mRNA and protein stabilisation [11–13]. Insulin-dependent diabetes mellitus has been shown to directly influence gene transcription and through the elevated levels of ketone bodies, particularly acetone, to influence protein stabilisation [14].

The pancreatic B-cell is particularly susceptible to nitroso compounds, such as streptozotocin, and to reactive intermediate damage [15]. This is believed to be a consequence of the low levels of protective antioxidant enzymes [16,17], within the pancreatic islets of Langerhans. As such, the presence of CYP2E1 within the pancreatic islets of Langerhans may have consequences for pancreatic B-cell function. Furthermore, a role for CYP2E1 in the metabolism of ketones via acetone to acetol and further metabolism to gluconeogenic precursors has been identified in animals [18]. As the pancreatic B-cell is responsive to circulating ketone body concentrations [19] it is possible that CYP2E1 may have a physiological role within the pancreatic B-cell.

The current investigation has examined the effect of induction of the cytochrome P450 CYP2E1 isoform on ketone-stimulated insulin release in rat pancreatic islets of Langerhans and also in mammalian pancreatic B-cell lines engineered for human CYP2E1 expression.

2. Methods

2.1. Materials

Collagenase (P) was purchased from Roche. Bovine serum albumin (BSA Fraction V), arachidonic acid, adrenaline, 5-bromo-4-chloro-3-indophosphate/nitroblue tetrazolium (BCIP/NBT), co-factors and general reagents were purchased from Sigma. Tissue culture and transfection reagents were purchased from Life Technologies. The human CYP2E1 vector, pSVh2E1, was a kind gift of Dr J. Doehmer, Technische Universität, Munich, Germany. The polyclonal goat anti-human CYP2E1 antibody for detection of CYP2E1 in cell lines and goat anti-rat NADPH-cytochrome P450 reductase antibody was purchased from Gentest. The polyclonal CYP2E antibody for detection of rat CYP2E was kindly provided by Dr. D. Koop, Cape Western University, USA.

2.2. Cell lines and cell culture

The parental BRIN BD11 cell line was produced by electrofusion of RIN5mF cell line with rat islet cells [20] and was cultured in RPMI medium supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°, 5% CO_2. INS-1 cells, established from cells isolated from an X-ray-induced rat transplantable insulinoma, were cultured in the same medium supplemented with 50 mM 2-mercaptoethanol, as this cell line is dependent on this chemical for continued growth [21]. These cells were kindly provided by Professor C.B. Wollheim, Geneva, Switzerland. Transfected cells were selected by incubation in the parental medium supplemented with 0.5 mg/mL G418 as determined by G418 killing curve. Culture continued at 0.2 mg/mL. Homologous clones were obtained by seeding cells at 1 cell per well in a 96-well plate and expanding. *In vitro* induction of CYP2E1 was carried out by supplementation of growth medium with 40 mM ethanol for 16 hr. This concentration of ethanol has been shown to induce maximum CYP2E1 activity in V79 Chinese hamster cells which stably express human CYP2E1 [22]. Two hours prior to experimentation, medium containing ethanol was replaced with fresh medium without ethanol to prevent competitive inhibition of CYP2E1.

2.3. Transfection

The parental BRIN BD11 and INS-1 cell lines were cotransfected with the recombinant human CYP2E1 expression vector, pSVh2E1 and the selectable pCIneo vector at a ratio of 50:1 using the cationic transfection reagent Lipofectamine Plus according to manufacturer's instructions. The recipient cell lines for the pSVh2E1 vector were named BRIN BD11h2E1 and INS-1h2E1 according to nomenclature for cytochromes P450 [23]. Cellular clones were screened for CYP2E1 expression by Western blotting.

2.4. Animals

Male Wistar albino rats (250–350 g) were obtained from the Biomedical and Behavioural Research Unit, University of Ulster. Animals were supplied with a standard pellet diet and water *ad libitum*. Induction of cytochrome P450

CYP2E1 was performed by treating the rats (N = 40) with acetone in drinking water (1%, v/v for 7 days). Control animals (N = 40) received normal drinking water. All animals were killed after 7 days and the pancreas and liver were excised from each animal and used for the following investigations.

2.5. Isolation of pancreatic islets of Langerhans

Islets of Langerhans were isolated from rat pancreata by collagenase digestion using a modification of the method of [24]. A collagenase-containing (1 mg/mL) HBSS solution (5.4 mM, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.63 mM Na_2HPO_4, 5.6 mM glucose) supplemented with 10 mM HEPES (*N*-2-hydoxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 5% (v/v) FBS, was used to distend the pancreas *in situ*, by injection into the common bile duct. The distended pancreas was removed and incubated at 37° for 40 min. 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 and intact islets were selected using a finely drawn glass micropipette under a binocular microscope.

2.6. Western blotting

Microsomal fractions of cell lines were prepared according to the method of [25]. Total microsomal protein concentration was determined using a Bio-Rad protein kit and comparison to a standard curve constructed from a range of known BSA standards. Cellular microsomal (70 μg) protein samples were separated by SDS/polyacrylamide gel electrophoresis using a modification of the method of [26]. Rainbow marker (Life Technologies) was included for confirmation of protein size. Following separation, the microsomal proteins were transferred to a nitrocellulose membrane [27]. The animal tissue membrane was probed for CYP2E1 protein using a polyclonal antibody (1:2000) and visualised using an alkaline-phosphatase linked secondary antibody and BCIP/NBT as substrate. Nitrocellulose blotted with microsomal protein from the control and transfected cell lines was probed for CYP2E1 protein using a goat anti-human CYP2E1 antibody (1:5000 dilution) or probed for NADPH-cytochrome P450 reductase using goat anti-rat NADPH-cytochrome P450 reductase antibody (1:5000) and a horse radish peroxidase (HRP) linked secondary antibody (1:30,000) and visualised using ECL detection reagents and exposure to Hyperfilm. Blots were converted to TIFF format and relative protein expression analysed using Phoretix 1-D advanced software.

2.7. CYP2E1 activity

CYP2E1 activity in transfected and control cell lines was determined by the microsomal hydroxylation of *p*-nitrophenol as described by [28] with modification by [29]. Essentially duplicate samples were prepared containing 0.13 M potassium phosphate buffer pH 6.8, 0.1 mM ascorbate, 0.1 mM *p*-nitrophenol, 5% (v/v) microsomal suspension containing approximately 300–500 μg total microsomal protein. The samples were incubated in a shaking water bath at 37° for 3 min before the addition of NADPH to a final concentration of 1 mM. After a further 10 min incubation the reaction was terminated and protein precipitated by the addition of ice-cold 0.6 N perchloric acid. The samples were centrifuged at 1000 *g* for 10 min. Sodium hydroxide was added to the supernatant to a final concentration of 1 M. The absorbance of each sample was read at 536 nm using a Phamacia NovaSpecII. The *p*-nitrophenol hydroxylase activity was calculated using an extinction coefficient of 10.28 mM^{-1} cm^{-1}.

2.8. NADPH-cytochrome P450 reductase activity

NADPH-cytochrome P450 reductase (EC 1.6.2.4) activity was measured in cell lines by the method of [30]. The reduction of cytochrome *c* electron acceptor was followed at 37° using the Pharmacia Biotech Ultraspec 2000 UV-Vis spectrophotometer. The microsomal suspension (300–500 μg) was diluted 1:40 with 30 mM potassium phosphate buffer, pH 7.6 containing 0.6 mM potassium cyanide and 0.033 mM cytochrome *c*. The reactants were preincubated at 37° and a steady baseline recorded. The reaction was initiated by the addition of 1 mM NADPH (in 1%, w/v sodium hydrogen carbonate). The reduction of cytochrome *c* followed at 550 nm by recording the increase in absorbance. The concentration of ferrous cytochrome *c* formed was calculated using an extinction coefficient of 18.5 mM^{-1} cm^{-1}.

2.9. Total cytochrome P450 determination

Microsomal cytochrome P450 content of each cell type was determined by the method of [31]. Total cytochrome P450 content of parental and transfected cells was measured as the difference spectrum of the reduced cytochrome P450-carbon monoxide bound complex vs. the reduced cytochrome P450 between 390–500 nm using the Shimadzu UV-210PC UV-Vis scanning spectrometer. Approximately 300–500 μg of total microsomal protein was diluted 1:40 with 0.1 M potassium phosphate buffer, pH 7.6 to give a total volume of 2 mL. Cytochrome P450 was reduced by the addition of sodium dithionite (approximately 5 mg) and the microsomal suspension divided between two cuvettes. A baseline was recorded between 390 and 500 nm. Carbon monoxide was bubbled through the test cuvette at a rate of two bubbles per second and the difference spectrum recorded. The absorbance difference of $A_{450\,nm} - A_{490\,nm}$ was calculated and an extinction coefficient of 91 mM^{-1} cm^{-1} used to determine the total P450 content of the cells.

2.10. Cytochrome b_5 determination

The method of [31] was used to determine the microsomal cytochrome b_5 content of the parental and transfected cell lines by recording the difference spectrum between the oxidised and dithionite-reduced forms of the cytochrome. Microsomal preparations containing approximately 300–500 μg total microsomal protein were diluted 1:40 with 0.1 M potassium phosphate buffer pH 7.6 to give a total volume of 2 mL. The microsomal suspension was divided between two cuvettes and a baseline recorded between 390 and 500 nm. Approximately 5 mg sodium dithionite was added to the test cuvette and the difference spectrum recorded under aerobic conditions. The absorbance difference of $A_{426\,nm} - A_{410\,nm}$ and an extinction coefficient of 185 $mM^{-1}\,cm^{-1}$ were used to determine the cytochrome b_5 content.

2.11. Insulin secretory responsiveness

For insulin secretion studies, monolayers of each cell line were seeded at a density of 2×105 cells per well in 24-well plates 24 hr prior to experimentation. Control cells (CTRL) were incubated in normal culture medium while pretreated cells (ETOH) were incubated overnight in medium supplemented with 40 mM ethanol. Two hours prior to experimentation, the ethanol containing medium was replaced with fresh medium to prevent competitive inhibition of CYP2E1. Prior to acute studies of insulin secretion, cell lines and freshly isolated islets were preincubated for 60 min at 37° in Kreb's Ringer Bicarbonate Buffer pH 7.4 (KRB; 115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2{\cdot}6H_2O$, 1.2 mM KH_2PO_4, 25 mM HEPES and 8.4% Na_2HCO_3) supplemented with 1.1 mM or 3 mM D-glucose, respectively, and 0.1% BSA. The buffer was then removed and the cells incubated with KRB buffer supplemented with 0.1% BSA and a range of stimulatory agents at 1.1, 3, 8.4 or 16.7 mM D-glucose. The secretagogues chosen are known to act at specific loci of the stimulus secretion coupling pathway of insulin release. At the end of the incubation period aliquots of the buffer were removed and stored at −20° for subsequent insulin assay.

2.12. Analysis

Insulin was measured by dextran charcoal radioimmunoassay [32], using guinea pig antiporcine insulin antiserum, crystalline rat insulin standard (Novo-Nordisk A/S, Bagsvaerd, Denmark) and ^{125}I-bovine insulin (Amersham International, UK). Insulin concentration was determined by means of the standard curve constructed from known standards using a spline fitting logarithm.

Statistical analysis was performed using SPSS version 11 software for WINDOWS (SPSS Inc.). Data are presented as mean ± standard error of the mean (SE mean). Two-way ANOVA was performed followed by LSD post hoc tests where there was no significant interaction. Where a significant interaction occurred data were subsequently analysed by one-way ANOVA followed by LSD post hoc tests. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. Expression of CYP2E1

Once established, homologous clones were screened for expression of CYP2E1 protein by Western blotting. This confirmed the presence of a protein with a relative molecular mass of approximately 55 kDa which was specifically labelled by goat anti-rat CYP2E1 antiserum displaying anti-human cross reactivity in both BRIN BD11h2E1 and INS-1h2E1 cell lines (Fig. 1a). This protein was absent in parental BRIN BD11 and INS-1 cell lines and the respective NEO controls. It can also be seen that overnight incubation of CYP2E1 expressing cells with ethanol resulted in higher expression levels of this apoprotein. These results were confirmed by densiometry analysis and by immunocytochemistry, which showed homogeneous fluorescence in CYP2E1 expressing cells which was increased on overnight incubation with 40 mM ethanol (unpublished observations). This fluorescence was absent in control parental and neo cells which did not express CYP2E1. NADPH-cytochrome P450 reductase protein was present (Fig. 1b) and active (Table 1) in all cell lines. Ethanol pretreatment did not cause a wide variation in NADPH-cytochrome P450 reductase expression or activity in either BRIN BD11 or INS-1 parental or neo controls. Treatment of animals with acetone was associated with increased levels of CYP2E1 within both liver and pancreatic islets of Langerhans (Fig. 1a).

3.2. Microsomal markers and CYP2E1 activity

A range of microsomal markers were assessed in the engineered cell lines to ensure that the transfection procedure had not affected these parameters. Low levels of total cytochrome P450 were observed in both parental and neo controls for both the BRIN BD11 and INS-1 cell lines (Table 1). Following engineering for CYP2E1 expression, total P450 levels in these cells were significantly higher in the BRIN BD11h2E1 cell line (0.272 ± 0.05 nmol/mg, $P < 0.001$) than the BRIN BD11 control (0.033 ± 0.04 nmol/mg), and this was further increased following culture with ethanol to 0.344 ± 0.03 nmol/mg ($P < 0.001$). Indeed, INS-1h2E1 also displayed a significantly higher level of total P450 than parental controls (0.200 ± 0.023 nmol/mg compared to 0.021 ± 0.005 nmol/mg, $P < 0.001$) and upon ethanol pretreatment the total P450 levels in these cells increased to 0.287 ± 0.003 nmol/mg ($P < 0.001$). Total cytochrome b5 levels were found to

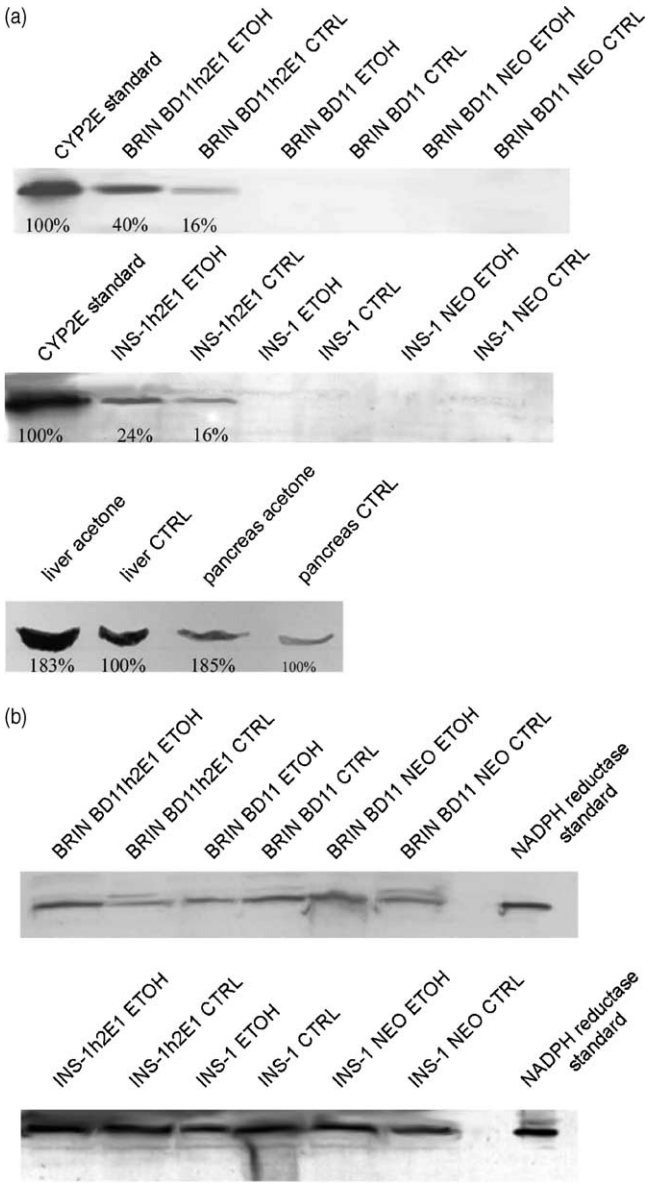

Fig. 1. Western blotting analysis of parental and transfected cell lines for (a) CYP2E1 and (b) NADPH-cytochrome P450 reductase proteins. Seventy micrograms of microsomal protein was loaded for detection of CYP2E1 or 40 μg for NADPH-cytochrome P450 reductase detection. CTRL, cells were not pretreated with ethanol or animals were not pretreated with acetone; ETOH, cells were incubated with 40 mM ethanol overnight; acetone, animals were preteated with acetone (1%, v/v for 7 days). Phoretix 1-D advanced software was used to measure relative band intensity (displayed below bands) by assigning an arbitrary value of 100% to each CYP2E standard (top and middle panel) or pancreas CTRL and liver CTRL (bottom panel).

be similar in all of the cell lines with no significant differences between parental, neo or CYP2E1 expressing cells, even following overnight culture with ethanol. Ethanol pretreatment had no effect on CYP2E1 activity in parental or neo control cells as evidenced by the lack of an appreciable difference in the rate of *p*-nitrophenol hydroxylation to 4-nitrocatechol (Table 2). The BRIN BD11h2E1 cell line displayed significantly higher rates of 4-nitrocatechol formation than the parental controls (0.341 ± 0.08 nmol/min/mg compared to 0.176 ± 0.08 nmol/min/mg, $P < 0.001$) and when pretreated with ethanol the activity increased to over 5-fold of the parental controls (0.986 ± 0.09 nmol/min/mg compared to 0.186 ± 0.05 nmol/min/mg, $P < 0.001$). This value represents a 3-fold increase on the same cells without ethanol pretreatment. The INS-1h2E1 *p*-nitrophenol hydroxylase activity was 0.633 nmol/min/mg which was over three times greater than the parental controls (0.204 nmol/min/mg, $P < 0.001$).

3.3. Insulin secretory response

The transfected cell lines displayed similar basal and stimulated secretory responsiveness to the parental cell lines when incubated with 1.1, 8.4 or 16.7 mM glucose (Fig. 2). Inclusion of 25 mM KCl, 25 μM forskolin, 10 nM

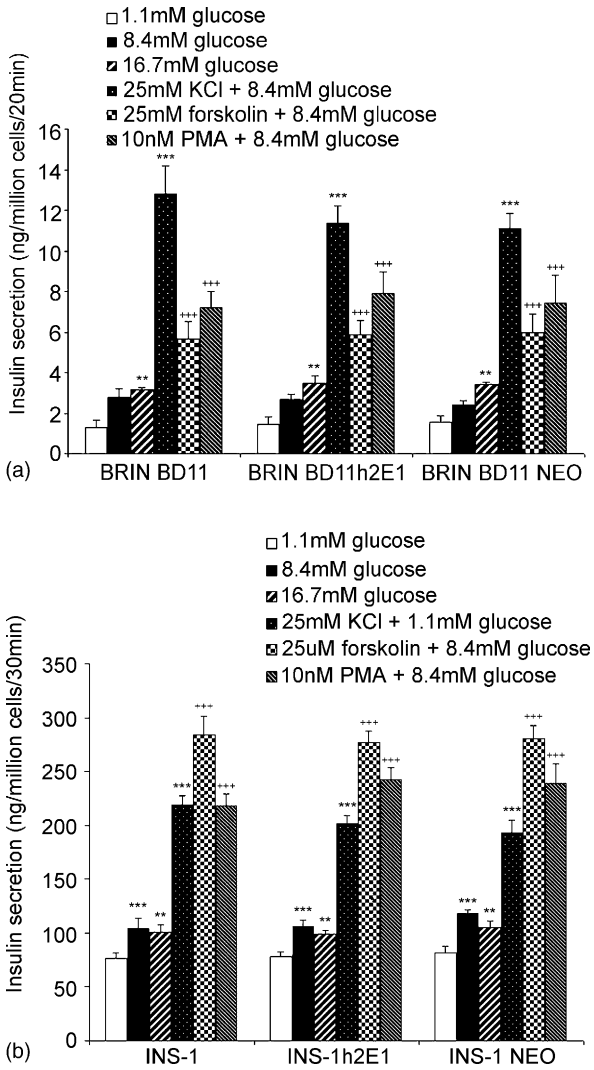

Fig. 2. Insulin secretion from (a) BRIN BD11 or (b) INS-1 parental and transfected cell lines in response to various secretagogues. Cells were seeded at a density of 2×10^5 cells per well and incubated overnight. Following preincubation cells were incubated with various secretagogues at 37° for 20 min (BRIN BD11 cell lines), 30 min (INS-1 cell lines) or 1 hr (islets). Values are mean ± SEM, N = 8. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 1.1 mM glucose alone (LSD test); $^{+++}P < 0.001$ compared to 8.4 mM glucose alone (LSD test).

Table 1
Levels of microsomal markers in BRIN BD11h2E1 and INS-1h2E1 cell lines

	Total P450 (nmol/mg/microsomal protein)	Total cytochrome b5 (nmol/mg/microsomal protein)	NADPH-cytochrome P450 reductase activity (nmol/min/mg microsomal protein)
BRIN BD11 CTRL	0.033 ± 0.04	0.135 ± 0.006	0.632 ± 0.023
BRIN BD11 ETOH	0.031 ± 0.02	0.122 ± 0.028	0.608 ± 0.041
BRIN BD11h2E1 CTRL	0.272 ± 0.05***	0.116 ± 0.087	0.657 ± 0.066*
BRIN BD11h2E1 ETOH	0.344 ± 0.03***	0.110 ± 0.024	0.824 ± 0.014*
BRIN BD11 NEO CTRL	0.087 ± 0.01	0.114 ± 0.041	0.627 ± 0.081
BRIN BD11 NEO ETOH	0.078 ± 0.02	0.104 ± 0.035	0.663 ± 0.028
INS-1 CTRL	0.021 ± 0.005	0.125 ± 0.036	1.568 ± 0.118
INS-1 ETOH	0.032 ± 0.003	0.105 ± 0.008	1.554 ± 0.062
INS-1h2E1 CTRL	0.200 ± 0.023***	0.100 ± 0.065	1.607 ± 0.093
INS-1h2E1 ETOH	0.287 ± 0.003***	0.150 ± 0.089	1.805 ± 0.066
INS-1 NEO CTRL	0.034 ± 0.025	0.113 ± 0.061	1.533 ± 0.011
INS-1 NEO ETOH	0.059 ± 0.008	0.134 ± 0.017	1.573 ± 0.099
Control rat liver microsomes	0.36 ± 0.04	0.61 ± 0.03	10.6 ± 0.4
2E-induced rat liver microsomes	0.55 ± 0.03	0.68 ± 0.03	14.5 ± 0.3

Values represent the mean of three independent experiments ± SEM. CTRL, cells cultured overnight in the absence of ethanol; ETOH, cells cultured overnight in media supplemented with 40 mM ethanol.
*$P < 0.05$, ***$P < 0.001$ compared to parental control (LSD test).

phorbol 12-myristate 13-acetate (PMA) in the test buffer resulted in 10-fold ($P < 0.001$), 2-fold ($P < 0.001$) and 2.5-fold ($P < 0.001$) increases in insulin secretion in the BRIN BD11 cell line compared with glucose alone. When incubated with these secretagogues, INS-1 cells showed a 2.8-fold ($P < 0.001$), 2.7-fold ($P < 0.001$) and 2-fold ($P < 0.001$) increase in insulin output. BRIN BD11h2E1, BRIN BD11 NEO, INS-1h2E1 and INS-1 NEO cell lines showed no differences in insulin secretion in response to these secretagogues compared to their parental cell line. Also overnight pretreatment of the cell lines with 40 mM ethanol and acute testing with 40 mM ethanol did not significantly alter the insulin secretory response of any of the cell lines (Fig. 3).

Table 2
CYP2E1 activity in parental BRIN BD11 and INS-1 cell lines, transfected BRIN BD11h2E1 and INS-1h2E1 cell lines

Sample	*p*-Nitrophenol hydroxylase activity (nmol/min/mg microsomal protein)
BRIN BD11 CTRL	0.176 ± 0.08
BRIN BD11 ETOH	0.186 ± 0.05
BRIN BD11h2E1 CTRL	0.341 ± 0.08***
BRIN BD11h2E1 ETOH	0.968 ± 0.09***
BRIN BD11 NEO CTRL	0.141 ± 0.03
BRIN BD11 NEO ETOH	0.186 ± 0.03
INS-1 CTRL	0.204 ± 0.03
INS-1 ETOH	0.195 ± 0.03
INS-1h2E1 CTRL	0.633 ± 0.10***
INS-1h2E1 ETOH	0.846 ± 0.10***
INS-1 NEO CTRL	0.150 ± 0.05
INS-1 NEO ETOH	0.143 ± 0.03
Control rat liver microsomes	0.9 ± 0.2
2E-induced rat liver microsomes	2.1 ± 0.2

Values presented represent the mean of three independent experiments ± SEM. CTRL, cells cultured overnight in the absence of ethanol; ETOH, cells cultured overnight in media supplemented with 40 mM ethanol.
***$P < 0.001$ compared to respective parental control (LSD test).

Acute incubation of control cell lines BRIN BD11, BRIN BD11 NEO, INS-1 and INS-1 NEO with the ketones 3-hydroxybutyrate (10 mM) and acetoacetate (15 mM) did not significantly increase insulin secretion (Fig. 4). Indeed no increase in insulin secretion was observed in CYP2E1 expressing cell lines which were not pretreated with ethanol. However, following pretreatment with ethanol, BRIN BD11h2E1 and INS-1h2E1 cell lines displayed 2.3-fold ($P < 0.001$) and 1.6-fold ($P < 0.001$) increases, respectively, when acute tested with 10 mM 3-hydroxybutyrate and 2.6-fold ($P < 0.001$) and 1.4-fold ($P < 0.001$) increases when acute tested with 15 mM acetoacetate.

Similarly, the insulin secretory responsiveness of islets isolated from control vs. acetone treated rats was determined in response to basal and stimulatory glucose and other established secretagogues. Incubation of islets from control rats with 16.7 mM glucose and 25 mM KCl elicited significant 3.7-fold ($P < 0.001$) and 6.8-fold ($P < 0.001$) increases in insulin output, respectively, compared to islets incubated with basal glucose alone (Fig. 5). Incubation of control islets with the ketone bodies, 10 mM 3-hydroxybutyrate or 15 mM acetoacetate, resulted in 1.9-fold

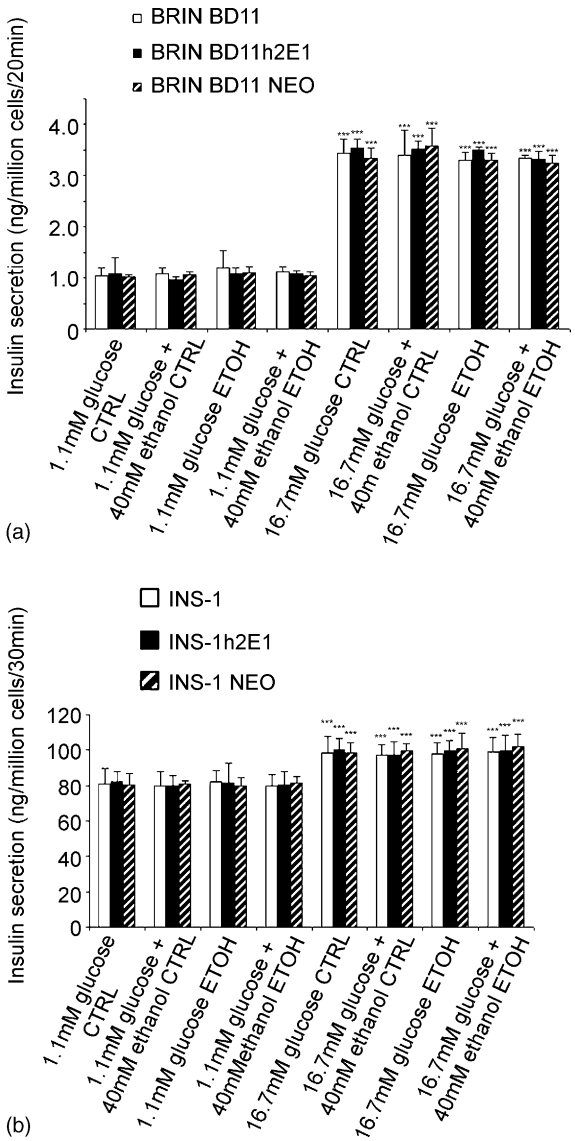

Fig. 3. Effects of 40 mM overnight ethanol pretreatment and acute incubation on (a) BRIN BD11 and BRIN BD11h2E1 cell lines and (b) INS-1 and INS-1h2E1 cell lines in response to 1.1 mM and 16.7 mM glucose. CTRL, no ethanol pretreatment; ETOH cells cultured with 40 mM ethanol overnight. Results are presented as mean ± SEM, N = 6. $^{***}P < 0.001$ compared to 1.1 mM glucose CTRL (LSD test).

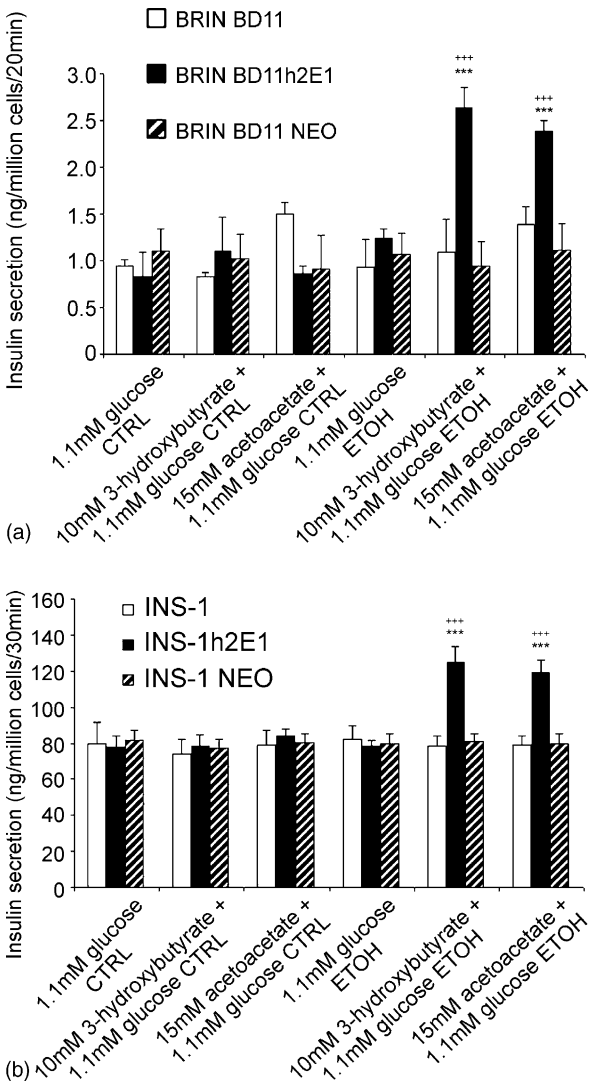

Fig. 4. Effects of 10 mM 3-hydroxybutyrate and 15 mM acetoacetate on insulin release from (a) BRIN BD11 and BRIN BD11h2E1 cell lines and (b) INS-1 and INS-1h2E1 cell lines following ethanol pretreatment. CTRL, no ethanol pretreatment; ETOH cells cultured with 40 mM ethanol overnight. Results are presented as mean ± SEM, N = 6. There was a significant interaction between cell line and secretagogoue ($^{a}P < 0.05$; $^{b}P < 0.001$) $^{***}P < 0.001$, compared to CTRL cells (LSD test); $^{+++}P < 0.001$ compared to 1.1 mM glucose alone (LSD test).

($P < 0.001$) and 1.6-fold ($P < 0.001$) increases, respectively in insulin output compared to basal glucose alone (Fig. 5). Similarly 10 nM PMA and 25 μM forskolin evoked significant 1.5-fold ($P < 0.001$), and 1.3-fold ($P < 0.001$) increases, respectively, in insulin secretion compared to islets incubated in 16.7 mM glucose alone (Fig. 5).

Islets derived from animals pretreated with acetone demonstrated similar basal and stimulated 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, and 25 μM forskolin (Fig. 5). However, islets from acetone treated rats demonstrated a significantly greater insulin secretory response to 10 mM 3-hydroxybutyrate and 15 mM acetoacetate (4.2 ± 0.3 ng/islet/hr and 3.5 ± 0.2 ng/islet/hr) compared to control islets (3.2 ± 0.3 ng/islet/hr and 2.8 ± 0.2 ng/islet/hr, respectively, $P < 0.05$).

4. Discussion

The CYP2E family of proteins have been demonstrated to be involved in the bioactivation of many low molecular weight carcinogens as well as drugs such as paracetamol. CYP2E1 is regulated at various points including increased gene transcription, mRNA and protein stabilisation depending on the chemical inducing agent [33]. Acetone and ethanol induce P450 through a common protein

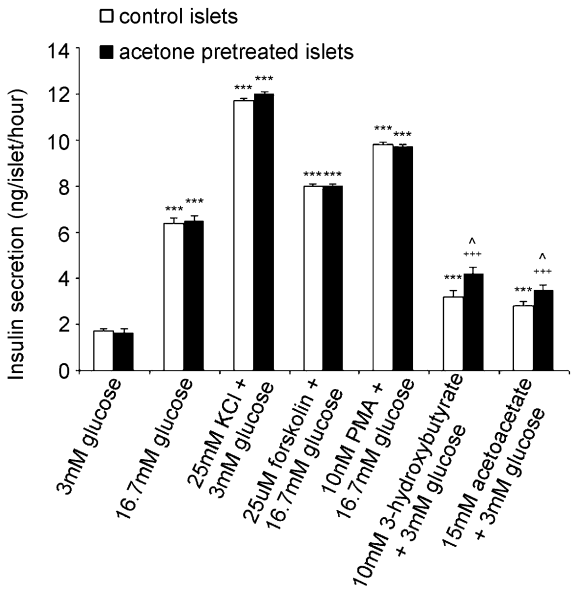

Fig. 5. Insulin secretory responsiveness of control and CYP2E1-induced islets in response to ketones. Islets were incubated at 3 mM glucose with 10 mM 3-hydroxybutyrate or 15 mM acetoacetate. Results are presented as mean ± SEM, N = 7. $^{***}P < 0.001$ compared to islets incubated with basal glucose (LSD test); $^{+++}P < 0.001$ compared to acetone pretreated islets in basal glucose (LSD test); $^{\wedge}P < 0.05$ compared to control islets (LSD test).

stabilisation mechanism. They both retain relatively high levels of CYP2E1 protein by binding close to or at the serine phosphorylating site inhibiting phosphorylation and subsequent loss of heme [34]. CYP2E1 has been demonstrated to be capable of metabolising acetone to acetol and further hydroxylation to molecules potentially capable of entering the gluconeogenic pathway [18]. As the pancreatic B-cell is responsive to circulating ketone bodies and permeable to many chemicals within the circulation, the expression of CYP2E proteins may have functional consequences. CYP2E1 protein is expressed and inducible within the pancreatic islets of Langerhans. These results have been confirmed by others [4,35]. We have also engineered two insulin secreting cell lines which express human CYP2E1 for further elucidation of the role of CYP2E1 in ketone-stimulated insulin release. Furthermore, it has been demonstrated in our laboratory and by others that the islets contain the NADPH-cytochrome P450 reductase protein [36]. Similarly, the bioengineered cell lines express NADPH-cytochrome P450 reductase thereby indicating the presence of functional cytochrome P450 systems in both pancreatic islets and our CYP2E1 expressing cell lines. Animals were pretreated with acetone while cell lines were pretreated with ethanol as acetone was found to be too toxic for *in vitro* use. Pretreatment of CYP2E1 transfected cell lines with ethanol resulted in increased expression of CYP2E1 as demonstrated by immunoblotting. However, control cell lines which were not transfected with the pSVh2E1 vector showed no expression of CYP2E1 or induction of this isoform when pretreated with ethanol.

Investigation of the effects of CYP2E1 induction on insulin secretory responsiveness was performed using a range of known nutrient and non-nutrient secretagogues. Pretreatment of animals or cell lines was not associated with increased insulin output. These findings suggest that the pretreatment did not cause alteration of cellular function *per se*. It was also established that the transfection procedure did not alter the stimulus secretion pathway of insulin secretion as determined by acute testing the cell lines with various secretagogues known to act at specific loci of the pathway [37]. Incubation with a range of increasing glucose concentrations showed a stepwise stimulation of insulin secretion in BRIN BD11 cell lines and isolated islets of Langerhans. The moderate response of the parental INS-1 cells to glucose was retained in both the INS-1h2E1 and INS-1 NEO cell lines. The values obtained correspond with data already published for INS-1 cells [21]. These findings indicate that islet and cellular glucose metabolism, glucose-induced closure of the ATP-sensitive potassium channels, and the rates of insulin biosynthesis and degradation were unaltered by pretreatment to induce CYP2E1 in islets and cell lines, respectively.

Acetone or ethanol pretreatment of islets or cell lines respectively with inducing agents did not result in any significant alteration of insulin secretory responsiveness compared to control islets and cell lines except in the case of ketone body stimulation. Acetoacetate and 3-hydroxybutyrate are the major forms of circulating ketone bodies with acetone being formed from the spontaneous decarboxylation of acetoacetate. Pretreated islets and CYP2E1 expressing cell lines demonstrated significant increases in insulin secretory responsiveness compared to control islets and cell lines when incubated with 3-hydroxybutyrate or acetoacetate. The mechanism of induction of insulin secretion is not fully understood but it has been suggested that ATP is generated by ketone metabolism within the pancreatic B-cell, thus stimulating exocytosis of insulin [19,38–42]. Due to the increase of insulin output in CYP2E1 induced islets and transfected cells it may be postulated that CYP2E1 may mediate the metabolism of ketones in these systems. This isoform has been shown to be active in the biotransformation of acetone *in vivo*, demonstrated by the concentration of blood acetone following administration of CYP2E1 inhibitors [43]. Also the blood acetone levels of male CYP2E1-null mice were significantly increased following 48 hr of fasting suggesting the inability of the mice to catabolize excess acetone produced during fasting [44]. CYP2E1 is the only P450 isoform which has been shown to be active in the conversion of acetone to acetol, an intermediate in the gluconeogenesis pathway [18,45]. Furthermore, CYP2E1 is the most active isoform in the further hydroxylation of acetol to 1,2-propanediol [18]. Also, a gluconeogenic pathway for acetone was elucidated by incorporation of [^{14}C]acetone into glucose and amino acids during fasting and diabetic ketoacidosis [46,47]. It has been shown that

CYP2E1-mediated metabolism of acetone is an important energy source for gluconeogenesis during prolonged fasting [44]. The insulin secretion studies presented here support the hypothesis that CYP2E1 may mediate metabolism of ketones to possible gluconeogenic precursors. The resultant generation of ATP from glucose metabolism is thought to elicit insulin release, thereby highlighting a possible role for CYP2E1 in pancreatic B-cell dysfunction. The concentrations of ketone bodies employed in this study are only found in individuals with uncontrolled diabetes, therefore the role of CYP2E1 in insulin secretion under normal physiological concentrations of ketones cannot be determined. CYP2E1 expression is associated with oxidative stress in cultured HepG2 cells which has been shown to alter the expression levels of other cellular proteins [48]. It cannot be ruled out that transfection of pancreatic cells with CYP2E1 may lead to upregulation of additional cellular proteins which could potentially mediate the enhancement of insulin secretion.

The induction of the CYP2E1 protein in this study employed specific inducing agents which bring about increased expression of CYP2E1 by a common mechanism. However, the CYP2E family of proteins are inducible by numerous environmental chemicals and drugs by various mechanisms. As such, induction of this family in the pancreatic islets of Langerhans by xenobiotic exposure could be envisaged to affect insulin secretion from the pancreatic B-cell with possible implications for the physiology and pathophysiology of insulin secretion.

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