Uncoupling of Nutrient Metabolism From Insulin Secretion by Overexpression of Cytosolic Phospholipase A₂

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We have generated MIN6 β-cells that stably overexpress cytosolic phospholipase A₂ (cPLA₂) and show a ninefold increase in cPLA₂ activity. Overexpression of cPLA₂ did not affect the capacity of MIN6 cells to show elevations in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in response to tolbutamide and KCl, and these depolarizing stimuli produced insulin secretion profiles in cPLA₂-overexpressing cells similar to those they produced in passage-matched nontransfected MIN6 cells. However, cPLA₂-overexpressing MIN6 cells did not respond to elevations in extracellular glucose with increases in ATP, [Ca²⁺]ᵢ, or insulin secretion. Nontransfected MIN6 cells showed a rapid and sustained increase in NAD(P)H autofluorescence in response to 25 mmol/l glucose, and this was reduced by ~95% in MIN6 cells overexpressing cPLA₂. This effect was mimicked in nontransfected MIN6 cells by p-(trifluoromethoxy) phenylhydrazone, a mitochondrial uncoupler. Quantitative RT-PCR indicated that mRNA for uncoupling protein-2 (UCP-2) was increased in the cPLA₂-overexpressing MIN6 cells, and this could be prevented by exposure to 100 μmol/l methyl arachidonyl fluorophosphate, a cPLA₂ inhibitor. Glucose caused a decrease in rhodamine 123 fluorescence in control cells, but not in those overexpressing cPLA₂, consistent with the transfected cells being unable to maintain mitochondrial proton gradients as a consequence of UCP-2 upregulation. Our data indicate that overexpression of cPLA₂ results in severe impairment of the calcium and secretory responses of β-cells to glucose through upregulation of UCP-2 and uncoupling of mitochondrial metabolism from ATP generation.

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COX, cyclo-oxygenase; cPLA₂, cytosolic phospholipase A₂; [Ca²⁺]ᵢ, intracellular Ca²⁺ concentration; FCCP, p-(trifluoromethoxy) phenylhydrazone; Kₛ,Kᵣ, channel, ATP-sensitive K⁺ channel; KIC, α-ketoisocaproate; MAPP, methyl arachidonyl fluorophosphate; [³H]IP, [³H]phosphatidylcholine 1α-stearyl-2-arachidonyl; UCP-2, uncoupling protein-2.

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of cPLA₂ were found to display no response to nutrient stimulation in terms of [Ca²⁺]i elevations and insulin secretion, although responses to depolarizing stimuli were largely unaffected. Because glucose-induced NAD(P)H generation was virtually abolished and UCP-2 levels were increased in these cells, we propose that elevations in endogenous arachidonic acid profoundly impair the secretory function of the β-cell, largely via upregulation of UCP-2 expression. This unexpected consequence of cPLA₂ overexpression may be relevant to lipotoxicity-induced failure of β-cell secretory responses.

RESEARCH DESIGN AND METHODS
MIN6 cells were obtained from Dr. Y. Oka and Prof. J.-I. Miyazaki (University of Osaka, Osaka, Japan). Tissue culture reagents, G418, and MMLV Murine leukemia virus reverse transcriptase were obtained from Life Technologies (Paisley, U.K.). We purchased a Dynabeads Oligo(dT)₂₅ kit from Dynal (Oslo, Norway). Restriction endonucleases and a CellTiter-Glo ATP quantification kit were obtained from Promega (Madison, WI), and pcDNA3.1 was from Stratagene Europe (Amsterdam, the Netherlands). PCR primers were prepared in house (Molecular Biology Unit, King’s College London), and RT-PCR was performed using LightCycler UCP-2 and cPLA₂ primers that were synthesized from Roche Diagnostics (Lewes, Sussex, U.K.). General laboratory chemicals, including fura-2 acetoxyethyl ester, phosphatidylcholine, arachidonic acid, rhodamine 123, 3-aminopropyltriethoxysilane, forskolin, and phorbol myristate acetate were purchased from Sigma (Poole, Dorset, U.K.). Anti-cPLA₂ antibody was purchased from Biogenesis (Poole), the secondary antibody was from Pierce (Rockford, IL), and [⁹⁸³H]phosphatidylcholine was from NEN DuPont (Herts, U.K.). An Axiovert 135 research microscope was obtained from Carl Zeiss (Welwyn Garden City, U.K.). Fluorescence spectroscopy was performed using a Perkin Elmer LS50B luminescence spectrometer (Beaconsfield, U.K.). A Veritas microplate luminometer was purchased from Turner Biosystems (Sunnyvale, CA).

Vector construction, transfection of MIN6 cells, and selection of clones. Cytosolic PLA₂ sense expression constructs were made by gel-purifying cPLA₂ cDNA and ligating it to pcDNA3.1, essentially as described for construction of antisense cPLA₂ vectors (18). MIN6 cells, grown in Dulbecco’s modified Eagle’s medium supplemented with 15% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/l glutamine, were electroporated (2 kV/cm, 3 μF) with [⁹⁸³H]phosphatidylcholine (1 mg/ml) and arachidonic acid (1 mmol/l), which was electroprecipitated (2 kV/cm, 3 μF) with HindIII-linearized cPLA₂ sense vector. Transfected cells were trypsinized and grown in medium supplemented with 1 mg/ml G418. Colonies of G418-resistant cells were expanded for analysis, and the clone that showed the highest expression of cPLA₂ was selected for functional characterization. Passage-matched, nontransfected MIN6 cells were used as controls in all experiments because these cells perform similarly to MIN6 cells stably transfected with empty pcDNA3.1 (18).

cPLA₂ expression and activity. The levels of expression of cPLA₂ were assessed by PAGE and immunoblotting, essentially as described (18), using polyclonal anti-cPLA₂ murine antibody and anti-mouse horseradish peroxydase-linked secondary antibody. cPLA₂ activity was measured as the release of [⁹⁸³H]arachidonic acid from the sn-2 position of [⁹⁸³H]phosphatidylcholine, as previously described for rat islets of Langerhans (19). Briefly, [⁹⁸³H]phosphatidylcholine (final concentration 2 µmol/l, specific radioactivity 20 Ci/mmol) was incubated with 2 mmol/l diethanolamine and 0.2% (vol/vol) β-mercaptoethanol to inhibit type I and type II PLA₂ activities and in the presence or absence of EGTA (1 mmol/l) or CaCl₂ (1 mmol/l) to inhibit or activate cPLA₂ activity, respectively. Reactions were terminated, and the lipid products were extracted by the addition of butanol (50 µl, 4°C) containing phosphatidylcholine (1 mg/ml) and arachidonic acid (1 mg/ml). The products in the butanol phase were separated by thin-layer chromatography on silica plates developed using petroleumether/diethylether/glacial acetic acid (70:30:1, vol/vol/vol). Products were visualized by iodine vapor, and [⁹⁸³H]arachidonic acid (Rt 0.62) was quantified by liquid scintillation spectroscopy.
Ca²⁺ microfluorimetry. Agonist-evoked changes in cytosolic Ca²⁺ were examined in MIN6 cells grouped within monolayer clusters (20–20 cells/cluster) on 3-aminopropyltriethoxysilane–coated glass coverslips, essentially as described previously (18).

Insulin secretion. We have shown previously that MIN6 cells show an improved secretory output when configured as pseudopotent structures rather than dissociated cells (20). We therefore carried out all secretion experiments using MIN6 cells configured as pseudosolts, using a dynamic multichamber perfusion system at 37°C, as described previously (18).

Real-time NAD(P)H autofluorescence. MIN6 cells adherent on 13-mm glass coverslips were incubated in glucose- and serum-free Dulbecco’s modified Eagle’s medium supplemented with 1% (wt/vol) BSA for up to 3 h. MIN6 cells adherent on 13-mm glass coverslips were washed twice with Krebs’ Ringer’s phosphate buffer (136 mmol/l NaCl, 4.7 mmol/l KCl, 1.25 mmol/l MgSO₄, 1.25 mmol/l CaCl₂, 5 mmol/l NaH₂PO₄, 2 mmol/l NaHCO₃, and 25 mmol/l HEPES, pH 7.2) and transferred to quartz cuvettes containing 3 ml Krebs’ Ringer’s phosphate buffer in an LS50B luminescence spectrometer. The cell-coated coverslips were maintained at 37°C and oriented across the diagonal of the cuvette so that the cell monolayer was facing the excitation light and light sharing away from the monitoring collection grating. Excitation was at 340 nm, and emission was measured as a trace at 450 nm. A 390-nm low-pass filter was included in the excitation channel to limit scattered light.

Real-time rhodamine 123 fluorescence. MIN6 cells were investigated according to the above protocol, except that rhodamine 123 at a final concentration of 25 µmol/l was added for the last 20 min of the 3-h preincubation period. Excitation was at 490 nm, and emission was measured as a trace at 590 nm, as described (21).

UCP-2 mRNA expression. Messenger RNA was isolated from control and cPLA₂-overexpressing MIN6 cells using a Dynabeads Oligo(dT)₂₅ kit and was reverse transcribed essentially as described previously (18). UCP-2 mRNA levels were quantified by real-time RT-PCR amplification using a LightCycler rapid thermal cycler system and expressed relative to β-actin mRNA levels in the same samples. Primers and probes were as follows: UCP-2 sense 5'-GGT GGA CAT ACC AGA GCA GCA CTT 3'; UCP-2 antisense 5'-GTG ACC TGC GCT GTG GTA CT 3'; β-actin sense 5'-AGC GCC AAG TCA TCA CTA TTG 3'; and β-actin antisense 5'-AGC CAC TGC TCC ACA CAG A 3'. The predicted sizes of the UCP-2 and β-actin PCR products were 308 and 300 bp, respectively. Real-time PCR was performed in a 10-µl volume essentially as described previously (18). All PCR protocols included a 10-s denaturation step and then continued for 40 cycles consisting of a 50°C denaturation for 0 s, annealing for 10 s at 55°C (β-actin) or 61°C (UCP-2), and a 72°C extension phase for 12 s (β-actin) or 13 s (UCP-2). Fluorescence measurements were taken at 83°C (β-actin) and 85°C (UCP-2) for 2 s to eliminate fluorescence from primer-dimer formation. The amplification products of both primer pairs were subjected to melting point analyses and subsequent gel electrophoresis to ensure specificity of amplification, and reaction products were cut from agarose gels, purified, and sequenced (18).

Glucose-induced ATP generation. The effect of glucose on the ATP content of control and cPLA₂-overexpressing cells was assessed using a luciferase-based assay. Cells were cultured in white-walled 96-well microtiter plates at a density of 2 × 10⁶ cells/well and pre-activated by incubation (2 h 37°C) in medium in the absence of glucose, followed by incubation (15 min, 37°C) in the presence or absence of a stimulatory concentration of glucose (20 mmol/l). ATP was extracted and measured using a CellTiter-Glo ATP quantification kit, according to the manufacturer’s instructions.

Data analysis. Data are expressed, where appropriate, as means ± SE and were analyzed statistically using Student’s t test, ANOVA, and Bonferroni’s multiple comparison test as appropriate. Differences between treatments were considered significant at P < 0.05.

RESULTS

CPLA₂ expression and activity in MIN6 cells transfected with a cPLA₂ expression vector. G418-resistant β-cell clones were expanded and harvested after transfection with the plasmid coding for cPLA₂, and Fig. 1A shows cPLA₂ expression in the clone that showed the highest cPLA₂ expression, compared with passage-matched control cells. This clone was used for all of the further experiments described in this article. The expression studies were confirmed by direct measurements of cPLA₂ enzyme activity in whole-cell extracts in which the Ca²⁺-dependent generation of [⁹⁸³H]arachidonic acid from phosphatidylcholine was determined. In control cells cPLA₂ activity was 220.3 ± 22.5 fmol·10⁶ cells⁻¹·10⁻⁶ cells⁻¹, and enzyme activity was markedly higher in extracts of stably transfected cPLA₂-overexpressing cells (945 ± 43% controls, P < 0.001, n = 4).

Tolbutamide and KCl-induced stimulus-response coupling in cPLA₂-overexpressing cells. Cytosolic
PLA<sub>2</sub> contains a calcium-binding C2 domain, so its overexpression could result in increased Ca<sup>2+</sup> buffering in the modified cells, thus potentially affecting the normal β-cell signal transduction cascades. We therefore initially examined the effect of depolarizing stimuli to generate increases in [Ca<sup>2+</sup>]<sub>i</sub> and elevations in insulin secretion. It can be seen from Fig. 1 that both cPLA<sub>2</sub>–stably transfected cells and passage-matched control MIN6 cells responded in a comparable manner to tolbutamide (100 μmol/l) and KCl (20 mmol/l). In these experiments 94% (44 of 47 cells in five experiments) and 99% (80 of 81 cells in nine experiments) of cPLA<sub>2</sub>-overexpressing cells showed increases in Ca<sup>2+</sup> in response to tolbutamide and KCl, respectively, compared with 97% (33 of 34 in four experiments) and 98% (58 of 59 in eight experiments) of control cells. The mean basal-to-peak change in cytosolic Ca<sup>2+</sup> did not differ significantly between test and control cells (cPLA<sub>2</sub>-overexpressing cells: tolbutamide 1.1 ± 0.61, n = 44; KCl 0.75 ± 0.34, n = 80; control cells: tolbutamide 0.81 ± 0.37, n = 33; KCl 1.20 ± 0.49, n = 58; P > 0.2).

Analysis of both the secretory responses to tolbutamide and KCl and the capacity of potentiators to enhance insulin secretion were carried out in a perifusion system. It can be seen from Fig. 2 that both KCl (Fig. 2A) and tolbutamide (Fig. 2B) significantly stimulated insulin secretion from MIN6 pseudoislets in which cPLA<sub>2</sub> had been overexpressed. However, the cPLA<sub>2</sub>-overexpressing cells failed to demonstrate an initial transient peak in secretion, and they showed a slower rise in secretory output that was not equal in magnitude to the control cells until 6–8 min after exposure to the stimuli. Tolbutamide- and KCl-induced secretory responses from both control and cPLA<sub>2</sub>–stably transfected cells responded in a comparable manner to tolbutamide (100 μmol/l) and KCl (20 mmol/l). In these experiments 94% (44 of 47 cells in five experiments) and 99% (80 of 81 cells in nine experiments) of cPLA<sub>2</sub>-overexpressing cells showed increases in Ca<sup>2+</sup> in response to tolbutamide and KCl, respectively, compared with 97% (33 of 34 in four experiments) and 98% (58 of 59 in eight experiments) of control cells. The mean basal-to-peak change in cytosolic Ca<sup>2+</sup> did not differ significantly between test and control cells (cPLA<sub>2</sub>-overexpressing cells: tolbutamide 1.1 ± 0.61, n = 44; KCl 0.75 ± 0.34, n = 80; control cells: tolbutamide 0.81 ± 0.37, n = 33; KCl 1.20 ± 0.49, n = 58; P > 0.2).

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overexpressing pseudoislets were potentiated by the adenylate cyclase activator forskolin (10 μmol/l) (Fig. 2A) and the protein kinase C activator phorbol myristate acetate (500 nmol/l) (Fig. 2B), with similar maximal secretory outputs obtained with control and cPLA2-overexpressing cells.

**Nutrient-induced stimulus-response coupling in cPLA2-overexpressing cells.** Although cPLA2-stably transfected cells and passage-matched control MIN6 cells showed similar Ca²⁺ responses to tolbutamide and KCl (Fig. 1), responses of the two sets of cells to nutrients were markedly different. Thus, as expected, sustained elevations in [Ca²⁺]i were observed in control MIN6 cell clusters in response to 20 mmol/l glucose or 10 mmol/l α-ketoisocaproate (KIC), a deamination product of leucine that directly enters the tricarboxylic acid cycle (17 of 28 and 30 of 32 cells responded to glucose and KIC, respectively, in four separate experiments) (Fig. 3B and D). However, the responsiveness to both glucose and KIC was dramatically reduced in cPLA2-overexpressing cells (2 of 52 and 3 of 40 cells responded to glucose and KIC, respectively, in 4–6 separate experiments) (Fig. 3A and C). The number of cells exhibiting glucose-induced increases in Ca²⁺ was increased in the presence of the cPLA2 inhibitor methyl arachidonyl fluorophosphate (MAFP; 18 of 53 cells in five experiments) (Fig. 3E), and the basal-to-peak change in Ca²⁺ in these cells was not significantly different from control cells (cPLA2-overexpressing + 50 μmol/l MAFP; 0.74 ± 0.31, n = 18; control: 0.57 ± 0.19, n = 17; P > 0.2).

Perfusion experiments indicated that control MIN6 pseudoislets showed a characteristic first phase of secretion followed by a lower sustained response at ~300% above basal levels in response to stimulatory levels of glucose (20 mmol/l). In contrast, cPLA2-overexpressing cells showed only a small, short-lived response to glucose, with glucose-induced secretion significantly less at all time points (P < 0.01 vs. control) (Fig. 4). The lack of secretion in response to 20 mmol/l glucose did not reflect insufficient insulin content, nor was it a consequence of abnormally elevated basal levels of insulin secretion because control and cPLA2-overexpressing cells had very similar insulin contents (sense: 1.24 ± 0.1 ng insulin/pseudoislet; control: 1.06 ± 0.06; n = 6–7, P > 0.1), and perfusion experiments indicated that overexpression of cPLA2 did not significantly affect the rate of insulin secretion at 2 mmol/l glucose (sense: 0.25 ± 0.01% insulin content; control: 0.26 ± 0.02%; n = 3, P > 0.2).

**Mitochondrial function in cPLA2-overexpressing cells.** The severely diminished calcium and insulin secretory responses to glucose, but maintained responsiveness to depolarizing agents, in cPLA2-overexpressing cells are consistent with impairment of the metabolic changes that lead to closure of ATP-sensitive K⁺ (KATP) channels. The absence of KIC-induced elevations in Ca²⁺ might suggest mitochondrial dysfunction, so oxidative metabolism was investigated in the control and cPLA2-upregulated cell populations using real-time NAD(P)H autofluorescence spectroscopy. Figure 5A shows that control MIN6 cells responded to 25 mmol/l glucose with a rapid and sustained rise in NAD(P)H autofluorescence, but in MIN6 cells overexpressing cPLA2 the capacity of glucose to elevate intracellular NAD(P)H was diminished by ~95%. Disruption of the mitochondrial membrane potential (ψm) was apparent from studies using the dye rhodamine 123 to dynamically monitor ψm. In control MIN6 cells, 25 mmol/l glucose elicited an increase in ψm, detected as a decrease in rhodamine 123 fluorescence caused by mitochondrial uptake and quenching of this dye (22). The addition of 10 μmol/l p-(trifluoromethoxy) phenylhydrazine (FCCP), an exogenous mitochondrial uncoupler, to control cells resulted in a rapid increase in rhodamine 123 fluorescence, consistent with dissipation of ψm and reduced dye quenching in the mitochondria. In MIN6 cells overexpressing cPLA2, the glucose-stimulated hyperpolarization of ψm was lost (Fig. 5B). The pattern of NAD(P)H autofluorescence in the presence of

**FIG. 2.** Effect of KCl and tolbutamide on insulin secretion from cPLA2-overexpressing MIN6 pseudoislets and passage-matched controls. MIN6 cells overexpressing cPLA2 (A) and passage-matched control (○) MIN6 pseudoislets were perifused with buffers, as shown by the horizontal bars. Data are means ± SE, n = 2–3 (A) and n = 4–6 (B).
A  
Glucose (20mM)  KCl (20mM)  
Estimate of cytosolic Ca\(^{2+}\) (ratio 340/380nm)  5min

B  
Glucose (20mM)  KCl (20mM)  
Estimate of cytosolic Ca\(^{2+}\) (ratio 340/380nm)  5min

C  
Tolb (100μM)  KIC (10mM)  KCl (20mM)  
Estimate of cytosolic Ca\(^{2+}\) (ratio 340/380nm)  5min

D  
Tolb (100μM)  KIC (10mM)  KCl (20mM)  
Estimate of cytosolic Ca\(^{2+}\) (ratio 340/380nm)  5min
25 mmol/l glucose in cPLA2-overexpressing cells could be reproduced by adding 10 μmol/l FCCP to control cells (Fig. 5C), providing circumstantial evidence supporting increased mitochondrial uncoupling after upregulation of cPLA2.

The possibility that chronic overexpression of cPLA2 in the MIN6 cells caused upregulation of the mitochondrial proton transporter UCP-2 was investigated by quantitative RT-PCR amplification of UCP-2 mRNA. These experiments indicated that mRNA for UCP-2 was significantly increased in the cPLA2-overexpressing MIN6 cells, and this could be reversed by 24-h exposure to 100 μmol/l MAFP, a cPLA2 inhibitor (Fig. 5D).

Glucose-induced ATP generation. Direct measurements of cellular ATP content confirmed a defect in glucose-induced ATP generation in the cPLA2-overexpressing MIN6 cells. Thus, in three separate experiments, glucose (20 mmol/l, 15 min) increased the ATP content of control cells (139.7 ± 11.2% basal, P < 0.01). In contrast, exposure to 20 mmol/l glucose did not increase ATP in the cPLA2-overexpressing cells, but rather caused a small decrease in their ATP content (93.0 ± 2.3% basal, P < 0.05).

DISCUSSION

One of the key events in stimulus-secretion coupling in response to elevations in extracellular glucose is an increase in [Ca2+]i (23), but the effector system(s) responsible for transducing this into an insulin secretory response has not been unequivocally established. An attractive candidate is cPLA2 because its activity in β-cells is regulated by micromolar levels of calcium (24). We have previously investigated the effects of underexpression of cPLA2 in MIN6 cells and found that significantly reducing levels of cellular cPLA2 had no effect on the cells’ ability to initiate a secretory response, although it compromised insulin synthesis and/or packaging (18). In the current study, we have investigated the effects of constitutive overexpression of cPLA2 in MIN6 cells by stable transfection with a vector housing the full-length coding sequence for cPLA2 driven by the cytomegalovirus promoter. Our measurements of cPLA2 immunoreactive protein and enzyme activity confirm a stable increase in the expression and function of cPLA2 in these cells.

It has recently been reported that short-term elevation in cPLA2 in mouse primary β-cells stimulated exocytosis, as assessed by changes in membrane capacitance (9). In those studies the stimulatory effects of cPLA2 were mimicked by arachidonic acid and lysophosphatidylcholine in an additive manner, and they were ascribed to priming of insulin secretory granules through enhanced Cl− uptake. The effects of chronic overexpression of cPLA2 on β-cell...
function described here are very different from those caused by transient (up to 24 h) upregulation (9). Thus, in our studies there was no circumstance under which insulin secretion was enhanced in \( \beta \)-cell populations stably overexpressing cPLA\(_2\). The secretory responses to depolarizing stimuli (tolbutamide and KCl) were of a similar magnitude to those observed in passage-matched control cells, but they were slower in onset, perhaps suggesting that excess cPLA\(_2\) has a restraining effect on the readily releasable pool of insulin. The most pronounced effect was the almost complete loss of insulin secretion in response to glucose by the cPLA\(_2\)-overexpressing MIN6 cells. Consistent with this, glucose only elicited increases in \([Ca^{2+}]_i\) in 4% of the overexpressing cells. The responsiveness to glucose could be partially restored by inhibiting cPLA\(_2\) activity with MAFP, suggesting that the elevated cPLA\(_2\) activity was responsible for failure of the cells to respond to glucose. It is unlikely that the high levels of arachidonic acid associated with elevated cPLA\(_2\) activity could have been responsible for the failure of the cells to respond to glucose with increases in \([Ca^{2+}]_i\), because arachidonic acid is known to stimulate increases in \(Ca^{2+}\) in \( \beta \)-cells (25,26). However, arachidonic acid is metabolized by cyclo-oxygenases (COXs), and it is known both that COX-2 levels are high in MIN6 cells (27) and that metabolism of arachidonic acid via the COX pathway results in opening of \(K_{ATP}\) channels (28). This could provide one mechanism by which overexpression of cPLA\(_2\) in \( \beta \)-cells could impair nutrient-induced elevations in \(Ca^{2+}\) without affecting responses to agents that directly close \(K_{ATP}\) channels or physically depolarize the cells.

Although our data indicate that an element of glucose-stimulated elevations in \(Ca^{2+}\) could be restored by inhibiting cPLA\(_2\) activity, the majority of cells in microfluorimetry experiments were still unresponsive to glucose, in terms of elevations in \(Ca^{2+}\), even in the presence of MAFP. This suggests that cPLA\(_2\) overexpression had produced additional perturbations of glucose-induced signaling that could not be reversed simply by short-term inhibition of cPLA\(_2\). Our observations that cPLA\(_2\)-overexpressing cells also failed to show elevations in \(Ca^{2+}\) in response to the mitochondrial substrate KIC suggest that the secretory defect lies downstream of glucose transport and glycolytic metabolism and places the focus on the mitochondrion as the locus of cPLA\(_2\)-induced cellular dysfunction.

It is well known that chronic exposure of \( \beta \)-cells to high
levels of fatty acids has a detrimental effect on normal cell function, resulting in reduced insulin secretory responses to nutrients but leaving responses to nonnutrients unaffected (12,14,15). There is also good evidence that prolonged exposure to fatty acids impairs mitochondrial function; reduced mitochondrial membrane potential and cellular ATP levels have been observed after 24- to 96-h exposure of β-cells to fatty acids such as palmitate and oleate (14,16). In addition, studies performed using INS-1 insulin-secreting cells have indicated that long-term (48–72 h) exposure to oleic acid increases both UCP-2 mRNA (14,17,29) and protein (14,29). The upregulation of UCP-2 is particularly relevant to β-cell (dys)function because it dissipates mitochondrial proton gradients, resulting in an insufficient membrane potential to drive ATP generation. It has been estimated that mitochondrial oxidative metabolism is responsible for up to 98% of the ATP that a β-cell produces (30), so interference with this process is likely to have deleterious effects on β-cell function.

These observations of UCP-2 upregulation fit well with the phenotype of the cPLA2-overexpressing MIN6 cells and could provide a feasible explanation for the severe impairment of nutrient-stimulated responses, with largely intact responses to tolbutamide and KCl. Although there are no reports on the effects of chronic exposure of β-cells to arachidonic acid, there is convincing evidence that arachidonic acid exposure results in increased UCP-2 mRNA levels in skeletal muscle (31) and hepatocytes (32). In the current study, mitochondrial dysfunction in the cPLA2-overexpressing MIN6 cells was indicated by a severely reduced increase in NAD(P)H autofluorescence upon exposure to 25 mmol/l glucose. In addition, cPLA2-overexpressing cells stained with rhodamine 123 did not exhibit an increase in ψm in response to glucose, in contrast to the clear response shown by passage-matched control cells. This is consistent with chronically elevated arachidonic acid uncoupling glucose metabolism from mitochondrial ATP generation, and it is further supported by our demonstration that exposure to stimulatory concentrations of glucose did not elevate the ATP content of cPLA2-overexpressing cells, in contrast to the expected increases in ATP seen in control cells.

In accordance with the measurements of NAD(P)H generation, ψm and ATP levels, real-time quantitative RT-PCR indicated that UCP-2 expression was significantly increased in cPLA2-overexpressing cells, providing a link between arachidonic acid excess and mitochondrial uncoupling. Thus, UCP-2 mRNA levels were increased almost 4-fold in the cPLA2-overexpressing cells and those overexpressing cPLA2 were maintained in the same ambient glucose concentration.

In conclusion, our data suggest that excessive levels of intracellular arachidonic acid generated through long-term constitutive expression of cPLA2 result in upregulation of UCP-2 and consequent mitochondrial uncoupling, thus greatly reducing the capacity of β-cells to respond to nutrients. This may well be relevant to β-cell failure, which can contribute to the onset of type 2 diabetes because chronic infusion of lipids rich in long-chain unsaturated fatty acids has recently been shown to reduce insulin secretion in individuals who are genetically predisposed to type 2 diabetes (37). Furthermore, MIN6 cells, in which intrinsically generated arachidonic acid has produced a selective loss of secretory responsiveness to nutrient stimuli, may offer a useful experimental model in which to further study the pathogenesis of this process.

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