INSULIN PROTECTS PANCREATIC ACINAR CELLS FROM CYTOSOLIC CALCIUM OVERLOAD AND INHIBITION OF THE PLASMA MEMBRANE CALCIUM PUMP

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Running Title: Insulin protects pancreatic acinar cells

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Background: Impaired metabolism and cytosolic Ca2+ overload in pancreatic acinar cells can trigger pancreatitis.
Results: Insulin protected cells from oxidant-induced Ca2+ overload, inhibition of the plasma membrane calcium pump (PMCA) and ATP depletion.
Conclusion: Insulin switches metabolism towards glycolysis and fuels the PMCA even when mitochondria are impaired.
Significance: This mechanism may provide an important therapeutic strategy for pancreatitis.

Acute pancreatitis is a serious and sometimes fatal inflammatory disease of the pancreas without any reliable treatment or imminent cure. In recent years impaired metabolism and cytosolic Ca2+ ([Ca2+]i) overload in pancreatic acinar cells have been implicated as the cardinal pathological events common to most forms of pancreatitis, regardless of the precise causative factor. Therefore, restoration of metabolism, and protection against cytosolic Ca2+ overload, likely represent key therapeutic untapped strategies for the treatment of this disease. The plasma membrane Ca2+-ATPase (PMCA) provides a final common path for cells to “defend” [Ca2+]i during cellular injury. In this paper we use fluorescence imaging to show for the first time that insulin treatment, which is protective in animal models and clinical studies of human pancreatitis, directly protects pancreatic acinar cells from oxidant-induced cytosolic Ca2+ overload and inhibition of the PMCA. This protection was independent of oxidative stress or mitochondrial membrane potential but appeared to involve the activation of Akt and an acute metabolic switch from mitochondrial to predominantly glycolytic metabolism. This switch to glycolysis appeared to be sufficient to maintain cellular ATP and thus PMCA activity, thereby preventing Ca2+ overload, even in the face of impaired mitochondrial function.

Acute pancreatitis is an inflammatory disease of the pancreas with an incidence of 1 in 10,000 and an overall disease-related mortality of 5%, which is considerably higher (17%) in severe necrotic disease (1). One-third of patients develop severe disease with in-patient stays of up to 100 days. There is no specific intervention for acute pancreatitis, and it is clear that current paradigms of understanding of the disease are inadequate. Although the underlying pathology is poorly understood, acute pancreatitis is often characterized by the autodigestion of the exocrine tissue, due either to impaired Ca2+-dependent exocytosis of digestive enzymes or to intracellular enzyme activation (1,2). This leads to a spiral of self-perpetuating necrotic tissue damage and a consequent local, and then systemic, inflammatory response. In severe cases this can lead to distal organ damage, multiple organ failure and death (1).

There are several diverse causative factors for pancreatitis, including bile acid reflux from gall stones or biliary disease, ethanol metabolites from excessive alcohol intake, hypertriglyceridaemia and oxidative stress (1,2). However, over the last several years, these causative factors have each been shown to impair the normal cytosolic Ca2+ concentration ([Ca2+]i)2 homeostasis and [Ca2+]i, signalling in pancreatic acinar cells (2). In particular, an irreversible increase in [Ca2+]i, (Ca2+ overload) has
been suggested to be a key feature of acute pancreatitis, regardless of the causative agent or process. Oxidative stress has also been implicated in pancreatitis, either as a cellular trigger (3) or in facilitating the inflammatory response (4).

We have previously reported that oxidative stress, induced by hydrogen peroxide (H$_2$O$_2$), profoundly altered hormone-evoked [Ca$^{2+}$], signalling and resulted in an irreversible Ca$^{2+}$ overload and a marked inhibition of the plasma membrane Ca$^{2+}$-ATPase (PMCA) in pancreatic acinar cells (5,6). Though oxidative stress can affect many Ca$^{2+}$ transport/signalling pathways, the PMCA has an especially key role as the final “gatekeeper” for the control of resting [Ca$^{2+}$], especially in cells in which the Na$^+$-Ca$^{2+}$ exchange (NCX) is not expressed (7). Even when all other Ca$^{2+}$ transport pathways are impaired, [Ca$^{2+}$], will recover close to resting levels as long as the PMCA remains active or “protected” (8). This will allow cells to recover from potential insults that raise [Ca$^{2+}$], by activating the necessary stress response pathways, or even triggering the “safe” dismantling of the cell constituents by apoptosis or autophagy (9). However, if the PMCA becomes inhibited, excess Ca$^{2+}$ in the cytosol cannot be exported and [Ca$^{2+}$], will remain high leading to catastrophic necrotic cell death. Therefore, understanding the mechanism for this inhibition of the PMCA and/or mechanisms by which the PMCA can be protected, could be an important basis for therapeutic strategies for acute pancreatitis, regardless of the precise causative factor or process.

Insulin, which is endogenously released from pancreatic β-cells adjacent to pancreatic acinar cells within the pancreas, has been reported to protect against pancreatitis, both in experimental animal models (10-13) and in the treatment of the human disease (14-16). For example, in L-arginine-induced experimental models of acute pancreatitis, most pancreatic acinar cells undergo damage, but acinar cells surrounding the islets of Langerhans remain relatively intact (10,11). This peri-insular (or peri-islet) acinar cell protection was abolished in streptozotocin-induced diabetic rats, where insulin secretion is impaired (10,11). Moreover, regeneration of exocrine pancreatic tissue was abolished in diabetic rats and restored following the administration of exogenous insulin (11-13). In addition, several related growth factors/GI peptides, that couple to similar signalling pathways to insulin (e.g. PI3K/Akt), have also been shown to be protective in several models of pancreatitis (17-19). Finally, activation of PI-3K/Akt signalling pathways has been extensively reported to protect a variety of cells from oxidative injury, activate pro-survival pathways and inhibit cell death pathways (20-22). The aim of the current study was therefore to test the protective effects of insulin on oxidant-mediated impairment of Ca$^{2+}$ signalling and inhibition of the PMCA. The results show that insulin protects against the oxidant-induced Ca$^{2+}$ overload and inhibition of the PMCA in a PI3K-dependent manner which correlated with Akt phosphorylation. Insulin had no effect on H$_2$O$_2$-induced oxidative stress or mitochondrial depolarisation, but appeared to reduce relative mitochondrial NADH production and enhance relative glycolytic NADH production. Insulin also attenuated the oxidant-induced ATP depletion, suggesting that this metabolic switch toward glycolysis was sufficient to maintain ATP. Moreover, insulin potentiated the inhibition of the PMCA by glycolytic inhibitors and abolished inhibition of the PMCA by mitochondrial inhibitors. This suggests that insulin may protect pancreatic acinar cells by switching from mitochondrial to predominantly glycolytic metabolism as the major ATP fuel for the PMCA, thereby maintaining low resting [Ca$^{2+}$] in the face of impaired mitochondrial function.

**EXPERIMENTAL PROCEDURES**

**Cell isolation**—Pancreatic acinar cells from Sprague Dawley rats were isolated by collagenase-digestion as previously described (5,6). For all fluorescence imaging experiments, cells were perfused with a HEPES-buffered physiological saline solution (HEPES-PSS; composition in mM: Na$^+$, 137; K$^+$, 4.7; Mg$^{2+}$, 0.56; Ca$^{2+}$, 1.28; Cl$^-$, 145.34; HEPES, 10; glucose, 5.5; pH 7.4). All drug solutions were made up from frozen stocks immediately prior to use.

**Imaging of Fura-2 Fluorescence**—Pancreatic acinar cells were loaded with 4 µM fura-2-AM (Invitrogen, Paisley UK) for 30 minutes at room temperature in HEPES-PSS as previously described (5). Fura-2-loaded cells were imaged using an identical microscope/imaging system to previous studies (5) and fura-2 fluorescence was calibrated into “estimated” [Ca$^{2+}$], as previously described (6). All experiments were carried out at room temperature (20-22°C).

**Imaging of dichlorofluoroscein (DCF) fluorescence**—Cellular oxidative stress was assessed using the fluorescent dye, dichlorofluoroscein (DCF), similar to our previous study (6). Pancreatic acinar cells were loaded with the non-fluorescent dichlorodihydrofluoroscein diacetate (H$_2$DCF-DA; Invitrogen, UK) at 10 µM for 30 min at room temperature. Cells were excited with light at 488 nm (± 10 nm) and background-
subtracted images were captured every 10 seconds through a fluorescein dichroic (510 nm; Chroma, USA) with 5x5 binning. The relative increase in DCF fluorescence was expressed as F/F₀ (where F₀ was the average initial fluorescence) and normalised to an extrapolated baseline following treatment with H₂O₂, with or without insulin.

Measurement of mitochondrial membrane potential (ΔΨm)—Mitochondrial membrane potential (ΔΨm) was determined using the fluorescent dye tetramethylrhodamine methylester (TMRM), as previously described (5). Pancreatic acinar cells were loaded with 100 nM tetramethylrhodamine methylester (TMRM) for 15 minutes at 37°C, and all perfusion solutions thereafter also contained 100 nM TMRM to minimize dye leakage. TMRM-loaded cells were excited with 545 ± 10 nm excitation light (50 ms exposure) and emitted fluorescence was captured through a TRITC dichroic (Chroma, VA, USA) every 5 seconds. Changes in relative TMRM fluorescence within mitochondrial regions were expressed as F/F₀. The effect of H₂O₂ on mitochondrial membrane potential was further normalised by expressing changes in F/F₀ as a percentage of the maximum depolarisation evoked by the uncoupler CCCP.

Western Blotting—Viable pancreatic acinar cells were separated from non-viable cells using Optiprep™ density gradient medium (Axis Shield). Acinar cell suspension was layered onto Optiprep™ at a density of 1.018 gml⁻¹ and centrifuged at 800g for 17 minutes at 4°C. The pellet containing non-viable cells was discarded, and the viable cells were washed in HEPES-PSS and re-suspended in 1% BSA. Cells were then treated with or without (control) test reagents (e.g. insulin (100nM) and/or LY294002 (10µM)). Following treatment, cells were lysed in RIPA buffer, sonicated and left to solubilise for 30 minutes on ice. RIPA buffer contained: 12mM HEPES, 300mM mannitol, 1mM EDTA, 1mM EGTA, 0.1mM Vanadate, 1mM NaF, 0.25mM Na₃P₂O₇, 1% Triton-X-100, 0.1% SDS and EDTA-free protease inhibitor tablet (Roche Diagnostics GmbH). Lysates were then centrifuged at 300 g for 15 minutes and the supernatant transferred into a fresh, ice cold eppendorf tube. Sample buffer containing β-mercaptoethanol was added at 1/3 supernatant volume and the mixture boiled at 95°C for 5 minutes. Proteins were separated using SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were then blocked in 5% BSA for 1 hour at room temperature. Following this, membranes were then washed and blotted with primary antibody (rabbit monoclonal anti-phospho-AktS473 or pan Akt antibody; Cell Signalling) at 4°C in 5% BSA either for 2 hours or overnight. Membranes were again washed and then probed with anti-rabbit secondary antibody for 1 hour at room temperature. Following a final wash, membranes were incubated in ECL for 3 minutes and then exposed to x-ray film and developed.

Imaging of NADH Autofluorescence—Pancreatic acinar cells were excited with light at 350 nm (500 ms exposure) and NADH autofluorescence collected through a fura-2 400 nm dichroic filter without a band pass filter. Sequential background-subtracted images were acquired every 5 seconds and changes in NADH autofluorescence quantified as raw fluorescence grey levels. To determine the relative mitochondrial and glycolytic contributions to NADH autofluorescence, cells were treated with 4 µM CCCP and then 2 mM iodoacetate (IAA), respectively.

Measurement of cellular ATP—ATP depletion in response to H₂O₂ (with or without insulin) was determined using two complimentary techniques. These were the Mg²⁺-sensitive fluorescent dye, magnesium green (MgGreen) used in intact living cells, and an in vitro chemiluminescence assay of fire-fly luciferase using cell extracts (ViaLight® Plus kit). MgGreen senses free [Mg²⁺], at concentrations around the resting [Mg²⁺], (~0.9mM) and was thus used as an indirect measure of cytosolic ATP depletion, similar to our previous study (5). This is because most cytosolic ATP exists as MgATP, and ATP depletion therefore increases free [Mg²⁺], and thus MgGreen fluorescence. Pancreatic acinar cells were incubated with 4 µM MgGreen for 30 minutes at room temperature and excited with 496 ±10 nm excitation light (50 ms exposure) every 10 seconds. Background-subtracted images were captured using 5x5 binning through a FITC dichroic (Chroma, VA, USA). Fluorescent signals were expressed as relative fluorescence normalised to fluorescence from the initial 10 frames (F/F₀). Cells were treated with or without 100 nM insulin for 20 minutes, followed by 500 µM H₂O₂ for a further 20 minutes before treatment with an ATP depletion cocktail (4 µM CCCP, 500 µM bromopyruvate, 10 µM oligomycin, 2 mM iodoacetate and 100 µM carbachol) for a final 20 minutes to induce maximum ATP depletion. All responses to H₂O₂ were normalised to the maximum ATP depletion following treatment with the ATP depletion cocktail, similar to our previous experiments.
The in vitro ViaLight® Plus kit (Lonza, Rockland, ME USA) was also used to assess cellular ATP. This measures the luminescence emitted from the fire-fly luciferase-catalysed ATP-dependent oxidation of luciferin, using a luminescence multi-plate reader (BioTEK Synergy HT). The kit was used according to the manufacturer’s instructions, with minor modifications due to the use of acutely isolated, rather than cultured, cells. Pancreatic acinar cells were treated with HEPES-PSS with or without 100 nM insulin for 20 minutes, followed by treatment with or without various concentrations of H₂O₂ (0-1 mM) for a further 20 minutes before treatment with or without the ATP depletion cocktail (for 20 minutes). Cells were aliquoted into eppendorf tubes and centrifuged between the above sequential treatments, prior to the final re-suspension in 100 µl Cell Lysis Reagent for 10 minutes. Each cell lysate was added to 100 µl ATP Monitoring Reagent Plus in a 96 well plate and left for 2 minutes prior to measuring the luminescence. All experimental treatment regimes were performed in parallel within the same experiment (i.e. on the same cell preparation) and all tubes were centrifuged at the same time to avoid the loss of cells during centrifugation from influencing the results. Each experiment also contained a time-matched control (TMC), in which cells received no drug treatment but were centrifuged at the equivalent time points, and thus represent total ATP. Finally, for ATP depletion, each experiment also contained a positive control in which an equal aliquot of cells was treated with the ATP depletion cocktail (during the final 20 min) which thus represented maximal/total ATP depletion. The total luminescence count of the ATP-depletion cocktail (max ATP depletion) was subtracted from the luminescence count from each corresponding assay condition prior to normalisation to the corresponding TMC (%) in each experimental run.

Data analysis and experimental design—Changes in resting [Ca²⁺], were quantified using both the maximum increase in [Ca²⁺] (magnitude) and area under the curve (AUC) and responses compared using an unpaired Student’s t-test. For [Ca²⁺]t clearance experiments, a paired experimental design was adopted, in which two repeated Ca²⁺ clearance phases were elicited and the normalised linear [Ca²⁺], clearance rate was compared to time matched control experiments using a Mann-Whitney test (5). For TMRM experiments, responses were normalised to the CCCP response and compared using a Mann-Whitney test. For each experimental manoeuvre there was a minimum of 4 separate experiments performed each containing 5-20 cells. For any given parameter analyzed, an “experimental average response” was determined from all the cells (5-20) in a particular experiment. These values were in turn averaged to give the true overall mean ± S.E.M.

RESULTS

Insulin protects pancreatic acinar cells

Insulin protects against H₂O₂-induced Ca²⁺ overload—In the present study we have tested the putative protective effect of insulin on the H₂O₂-induced Ca²⁺ overload response in pancreatic acinar cells. The nature of the experimental design and analytical methods are described in more detail in our previous work (5). Pancreatic acinar cells were pre-treated with or without 100 nM insulin for 30 minutes, as this concentration is known to cause maximum activation of glucose uptake in insulin responsive tissues such as skeletal muscle and adipocytes (23-25). Cells were then treated with 100 µM H₂O₂ (which approximates to the EC₅₀ value for H₂O₂ (6)) for 10 minutes followed by a further recovery period of 10 minutes. These experiments were repeated with or without 30 minutes pre-treatment with 100 nM insulin (see Figure 1). Responses were quantified by measuring both the maximum H₂O₂-induced change in resting [Ca²⁺], (max Δ Ca²⁺, figure 1D) and the area under the curve (AUC, figure 1E) for the [Ca²⁺], response. The AUC reflects both the magnitude and any recovery of the response, and was therefore measured over a 20 minute period that consisted of 10 minutes of H₂O₂ treatment and 10 minutes of recovery (see Figure 1).

On average, 100 µM H₂O₂ increased [Ca²⁺], by 235 ± 57 nM (n=15), which was reduced to 54 ± 6 nM (n=9, p<0.05 vs control cells) in the presence of insulin. Likewise, the H₂O₂-induced increase in area under the curve (AUC; 2.05 ± 0.4 µM.s, n=15) was also markedly attenuated in insulin-treated cells (0.55 ± 0.1 µM.s, n=7, p<0.05). When applied to pancreatic acinar cells alone, insulin (100 nM to 10 µM) had no direct effect on resting [Ca²⁺]t (see inset figure 1Bi), suggesting that even at higher concentrations insulin does not cause cytotoxicity over this time scale.

Insulin protection against H₂O₂-induced Ca²⁺ overload is prevented by LY294002—To test whether the protective effects of insulin were due to activation of PI3K/Akt pathways, cells were treated with the PI3K inhibitor LY294002 (10µM). When applied alone, LY294002 had no effect on resting [Ca²⁺]t (see inset figure 1Ci), suggesting that inhibition of any constitutively active PI3K has no
effect on resting \([\text{Ca}^{2+}]\). However, the combined treatment of cells with LY294002 (10µM) and insulin (100 nM) abolished the protective effect of insulin (figure 1C, 1D and 1E).

**Insulin has no effect on H\(_2\)O\(_2\)-induced oxidative stress**—Cells exhibited minimal fluorescence until treated with H\(_2\)O\(_2\), which oxidised the non-fluorescent H\(_2\)DCF to the fluorescent DCF. In some cells there was a slight upwards drift in DCF fluorescence, probably due to endogenous cellular oxidant production. The change in relative fluorescence (F/F\(_0\)) induced by H\(_2\)O\(_2\) was therefore calculated from an extrapolated linear baseline. H\(_2\)O\(_2\) (0.1-1 mM) caused a concentration dependent increase in relative DCF fluorescence (0.1mM H\(_2\)O\(_2\): \(\Delta F/F_0 = 19 \pm 4\%\) (n=4), \(p < 0.05\); 0.5mM: \(\Delta F/F_0 = 32 \pm 10\%\) (n=6), \(p < 0.05\); 1mM: \(\Delta F/F_0 = 31 \pm 6\%\) (n=8), \(p < 0.05\); see Figure 2A & C). Pretreatment of cells with 100 nM insulin for 30 minutes had no significant effect on the H\(_2\)O\(_2\)-induced increase in oxidative stress (0.1mM: 10 ± 2 % (n=3); 0.5mM: 35 ± 5 % (n=6); 1mM: 24 ± 6% (n=8); see Figure 2B & C). When applied alone, 100 nM insulin had no effect on DCF fluorescence (data not shown). These data suggest that insulin did not protect pancreatic acinar cells by reducing the H\(_2\)O\(_2\)-induced cellular oxidative stress, for instance by increasing cellular antioxidant capacity.

H\(_2\)O\(_2\) causes a concentration-dependent inhibition of the PMCA—To test whether the protective effect of insulin on H\(_2\)O\(_2\)-induced Ca\(^{2+}\) overload was due to protection of PMCA activity, we utilised an *in situ* \([\text{Ca}^{2+}]\) clearance assay similar to our previous study (5). In essence, this approach controls for both cell-to-cell, and time-dependent, differences in \([\text{Ca}^{2+}]\), clearance rate by making repeated measurements of \([\text{Ca}^{2+}]\), clearance, in parallel, on cells from the same experimental animal. This then allows changes in clearance/PMCA activity due to experimental treatments (H\(_2\)O\(_2\), insulin, LY294002) to be clearly identified. Cells were treated with cyclopiazonic acid (CPA) in the absence of external Ca\(^{2+}\); this inhibits the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), facilitates the net leak of Ca\(^{2+}\) from the ER and thus slowly depletes the ER of Ca\(^{2+}\). This manoeuvre causes a slow increase in \([\text{Ca}^{2+}]\), which slowly recovers to baseline due to Ca\(^{2+}\) efflux via the PMCA. Due to the ER Ca\(^{2+}\) depletion and the consequent switching on of store-operated Ca\(^{2+}\) entry (SOCE) channels, addition of high external Ca\(^{2+}\) (20 mM) to cells under these conditions causes a rapid increase in \([\text{Ca}^{2+}]\), which reaches a short-lived steady state due to a balance of Ca\(^{2+}\) entry and Ca\(^{2+}\) efflux. Subsequent removal of external Ca\(^{2+}\) (0 Ca\(^{2+}\), 1 mM EGTA) causes a rapid clearance of \([\text{Ca}^{2+}]\), back to resting values, due predominantly to PMCA activity (Figure 3). As previously described (5), this \([\text{Ca}^{2+}]\), clearance can be quantified by either fitting the falling phase to a single exponential decay or by measuring the linear rate from a standardised value of \([\text{Ca}^{2+}]\) (see our previous study (5)). However, fitting to a single exponential decay becomes unreliable when clearance is inhibited to the extent that only a very slow clearance rate is measurable. Under these circumstances the rate of clearance is quasi-linear, and therefore the linear rate of clearance was chosen for quantification.

Using this approach, the second clearance rate (R2) was found to be on average 92 ± 12 % of the initial clearance rate (R1) in time-matched control experiments (n=8). Treatment with H\(_2\)O\(_2\) caused a concentration-dependent inhibition of this relative \([\text{Ca}^{2+}]\), clearance rate (50 µM, 46 ± 10 %, n=4, \(p<0.05\); 100 µM, 32 ± 6 %, n=6, \(p<0.05\); 500 µM, 12 ± 5 %, n=6, \(p<0.05\); see figure 3), in accordance with our previous work (5).

**Insulin protects against H\(_2\)O\(_2\)-induced inhibition of the PMCA in a PI3K-dependent manner**—We next tested whether insulin protected this H\(_2\)O\(_2\)-induced inhibition of the PMCA. A single concentration of 100 µM H\(_2\)O\(_2\) was chosen, as this was close to the IC\(_{50}\) value for PMCA inhibition (Figure 3). In insulin-treated cells, 100 µM H\(_2\)O\(_2\) reduced \([\text{Ca}^{2+}]\), clearance to 57 ± 3 % (Figure 4B; n=6) of control, demonstrating less inhibition of \([\text{Ca}^{2+}]\), clearance by H\(_2\)O\(_2\) than in untreated control cells (32 ± 6 %, figure 4A; n=6, \(p<0.05\)). This suggests that insulin partially protects the PMCA from inhibition by oxidative stress. To test whether this protection was due to activation of the PI3K/Akt pathway, we again incubated cells with the PI3K/Akt inhibitor LY294002 (10 µM) in combination with 100 nM insulin (Figure 4C, D and E). This restored the full inhibitory effect of H\(_2\)O\(_2\) on \([\text{Ca}^{2+}]\), clearance (reduced to 30 ± 5 % of control, n=5, Figure 4E). These data collectively suggest that insulin protects the PMCA from oxidative stress due to activation of PI3K pathways.

**Insulin causes a PI3K-dependent Akt phosphorylation in pancreatic acinar cells**—We next verified that treatment of pancreatic acinar cells with insulin activates insulin-dependent PI3K/Akt by western blotting using the pAktS473 antibody (see Figure 5). This detects the phosphorylated serine residue at position 473 on Akt and is routinely used as a convenient indirect measure of Akt activation (21). Pancreatic acinar
cells were treated with or without insulin in the absence or presence of LY294002 for 15 minutes. Cells were then lysed and protein lysates were separated using SDS PAGE and western blotted using the pAktS473 antibody (Figure 5, top panel). The pan-Akt antibody was used in parallel control experiments to determine whether equal amounts of protein (and specifically of Akt) were loaded into each lane (Figure 5, bottom panel). Treatment with 100 nM insulin increased Akt phosphorylation, which was completely inhibited by co-incubation with the PI3K inhibitor, LY294002 (Figure 5, top panel). In fact, LY294002 reduced Akt phosphorylation to well below control levels, suggesting that there may be some constitutive Akt phosphorylation in resting cells. However, the lack of effect of LY294002 on resting [Ca\(^{2+}\)], suggests that reducing this constitutive Akt phosphorylation (with LY294002) has no effect on [Ca\(^{2+}\)]\(_{\text{i}}\) homeostasis.

**Insulin has no effect on H\(_2\)O\(_2\)-induced mitochondrial depolarisation**—A variety of evidence suggests that some of the major downstream targets of Akt reside at or within the mitochondria (26-28). Several of these molecular targets are either components of, or can regulate the activity of, the mitochondrial permeability transition pore (mPTP). Our previous study showed that oxidant-induced inhibition of the PMCA coincided with mitochondrial depolarisation, and that both phenomena were prevented by inhibitors of the mPTP (5). This therefore suggested that the mechanism for the protective effect of insulin on Ca\(^{2+}\) overload and inhibition of the PMCA in pancreatic acinar cells might be due to protection of the mPTP and prevention of H\(_2\)O\(_2\)-induced mitochondrial depolarisation. We tested this using tetramethyl rhodamine methylester (TMRM) to measure H\(_2\)O\(_2\)-induced mitochondrial depolarisation. H\(_2\)O\(_2\) induced a slow mitochondrial depolarisation, while the protonophore and mitochondrial uncoupler, CCCP (4 µM), induced a rapid and almost complete mitochondrial depolarisation (Figure 6A). Surprisingly, H\(_2\)O\(_2\) induced on average a 45 ± 6 % (Figure 6B and D; n=6 cells) depolarisation in insulin-treated cells, which was indistinguishable from untreated control cells (51 ± 6 %; n=6 cells; Figure 6A and D). Moreover, insulin had no effect on TMRM fluorescence when applied to cells alone (Figure 6C and D). Collectively, these data suggest that protection against mitochondrial depolarisation is unlikely to be the mechanism whereby insulin protects against oxidant-induced inhibition of the PMCA.

**Insulin attenuates the H\(_2\)O\(_2\)-induced ATP depletion**—Since insulin had no effect on mitochondrial depolarisation, we next wanted to test whether insulin could protect against ATP depletion. This was investigated using two complimentary approaches; MgGreen fluorescence in intact living cells and in vitro chemiluminescence of fire-fly luciferase assays. MgGreen was used to assess ATP depletion indirectly by measuring free [Mg\(^{2+}\)], as in our previous study (5). Using the MgGreen technique, 500 µM H\(_2\)O\(_2\) caused 50 ± 3 % ATP depletion (Figure 7A and C), consistent with our previous study (5). However, following treatment with 100 nM insulin, the response to 500 µM H\(_2\)O\(_2\) was reduced to 15 ± 3 % ATP depletion (Figure 7B and C).

For experiments using the in vitro fire-fly luciferase chemiluminescence ATP assays kits, the experimental design was essentially the same, except cells were treated sequentially in suspension in eppendorf tubes and centrifuged between treatments, prior to cell lysis and assay of luminescence. A range of H\(_2\)O\(_2\) concentrations were used to give a full H\(_2\)O\(_2\) concentration-ATP depletion response curve, in the absence or presence of insulin. These experiments revealed that H\(_2\)O\(_2\) caused a steep concentration-dependent ATP depletion between 30 µM, at which there was no significant ATP depletion, and 300 µM, which reached close to maximum ATP depletion (see figure 7D). The data were fitted to log transformed sigmoidal concentration-response curves which generated an average IC\(_{50}\) of 86 ± 8 µM and Hill slope of 3.8 ± 1.3 (n=9 assays, 5 rats; Figure 7D, filled square). Pre-treatment of cells with insulin (100 nM) caused a rightward shift in the concentration-response curve and significantly increased the average IC\(_{50}\) to 254 ± 42 µM (p<0.0001; Hill slope of 2.7 ± 1.2; n=9 assays, 5 rats; Figure 7D, open circle). Surprisingly, insulin treatment alone (without H\(_2\)O\(_2\)) for the entire experimental period (3x20 minutes) had no significant effect on ATP levels compared to time-matched controls (105 ± 8 %; n=9 assays, 5 rats; Figure 7D, open triangle). These data suggest that insulin protects acinar cells from substantial ATP depletion over a relatively narrow concentration range of H\(_2\)O\(_2\).

**Effect of insulin on NAD(P)H autofluorescence in pancreatic acinar cells**—To test whether insulin causes a metabolic switch from mitochondrial to predominantly glycolytic metabolism in pancreatic acinar cells, we next used NAD(P)H autofluorescence as an indirect measure of cellular...
metabolism. The major pool of cellular NAD(P)H is classically believed to be located within mitochondria whereas a minor pool comes from glycolytic enzymes in the cytosol. Therefore to determine the relative contributions of mitochondrial versus cytosolic (glycolytic) NAD(P)H production, cells were treated with CCCP (4 µM) to deplete mitochondrial NAD(P)H, followed by iodoacetate (IOA, 2 mM) to inhibit cytosolic (glycolytic) NAD(P)H production. The protonophore, CCCP, uncouples mitochondrial electron transport from ATP production by dissipating the electrochemical proton gradient, thereby allowing mitochondrial reducing equivalents (NAD(P)H) to be used up unproductively (29). IOA is a potent and irreversible inhibitor of glyceraldehyde phosphate dehydrogenase, which is the major glycolytic source of NADH (30). Therefore, the CCCP-induced decrease in NAD(P)H autofluorescence represents an indirect measure of mitochondrial metabolism; while the residual IOA-induced decrease in NAD(P)H autofluorescence represents an indirect measure of glycolytic metabolism. Similar methods have been used to assess the relative mitochondrial and glycolytic contribution to NADH production, and thus metabolism, in transformed hematopoietic cells (31). The relative CCCP and IOA-induced changes in NAD(P)H autofluorescence were quantified and normalised by expressing them as a percentage of the total decrease in NAD(P)H autofluorescence (Figure 8). In untreated control cells CCCP caused a 55 ± 4 % decrease, and IOA a 45 ± 4 % decrease, in NAD(P)H autofluorescence (Figure 8A & C; n=6). However, in cells pre-treated with 100 nM insulin for 30 minutes, the CCCP-induced decrease in NAD(P)H autofluorescence was markedly reduced to only 17 ± 4 % (Figure 8B & C; n=7, p<0.05, assessed by Mann Whitney test), whereas the IOA-induced decrease in NAD(P)H autofluorescence increased to 83 ± 4 % (Figure 8B & C; n=7, p<0.05). These data suggest that insulin treatment switches pancreatic acinar cell metabolism from mitochondrial to predominantly glycolytic metabolism.

Insulin potentiates the inhibition of the PMCA by glycolytic inhibitors and abolishes the inhibition of the PMCA by mitochondrial inhibitors—Due to this insulin-induced metabolic switch, we reasoned that insulin may also affect the sensitivity of the PMCA to the glycolytic inhibitor, 3-bromopyruvate (BrPyr), versus the mitochondrial uncoupler, CCCP. BrPyr is an inhibitor of hexokinase, the first step in the glycolytic pathway. Similar to experiments performed in Figure 4, cells were treated with or without 100 nM insulin for 30 minutes prior to the start of the [Ca^{2+}]i clearance assay. Each metabolic inhibitor was then applied during the second clearance phase and its effect determined on the normalized clearance rate. In the absence of insulin treatment, BrPyr (500 µM) reduced [Ca^{2+}]i clearance to 45 ± 8 % of the initial clearance rate (n=7, figure 9A and E) compared to time-matched control experiments (103 ± 3 %, n=6). However, in insulin-treated cells the BrPyr-induced inhibition of [Ca^{2+}]i clearance was markedly potentiated to 12 ± 8 % of the initial clearance rate (n=8, figure 9B and E; p<0.05). Likewise CCCP (4 µM) decreased [Ca^{2+}]i clearance to 58 ± 10 % of the of the initial clearance rate in untreated control cells (n=8, figure 9C and E), which was abolished to 115 ± 19 % following insulin treatment (n=8, figure 9D and E). These data suggest that insulin treatment causes the PMCA to become almost entirely dependent on glycolysis as an ATP supply.

DISCUSSION

We have previously demonstrated that increased oxidative stress (H2O2) alters the normal pattern of CCK-evoked [Ca^{2+}]i signalling and impaired normal resting [Ca^{2+}]i homeostasis. This resulted in an irreversible increase in [Ca^{2+}]i (Ca2+ overload response) in an increasing proportion of cells with increasing concentration of H2O2 (6). The H2O2-induced Ca2+ overload we observed also corresponded to a marked inhibition of the PMCA (5). This inhibition is likely to be an important mechanism responsible for the irreversible nature of the Ca2+ overload response because pancreatic acinar cells do not express Na+-Ca2+ exchanger (NCX) or show functional NCX activity (7) and the PMCA is thus the only Ca2+ efflux pathway. Even if other Ca2+ clearance pathways and [Ca2+], signalling are impaired, provided the PMCA remains functional, [Ca2+], will slowly recover. This can be seen, for instance after cells are treated with inhibitors of SERCA, such as CPA. In the context of a cellular insult, as in pancreatitis, this gives the cell time to activate and upregulate appropriate stress response pathways (32) or even allows the “safe dismantling” of the cell constituents by activation of apoptosis or autophagy (33).

The current study showed that the H2O2-induced Ca2+ overload response and inhibition of the PMCA was partially prevented by pretreatment with insulin. This functional protection by insulin was abolished by the PI3K inhibitor, LY294002, suggesting that the protection was due to activation of PI3K, and was accompanied by Akt phosphorylation and thus activation. Protection by insulin was, however, independent of reduced
Insulin protects pancreatic acinar cells

oxidative stress or any protective effect of insulin on mitochondrial depolarisation. Nevertheless, insulin attenuated the H$_2$O$_2$-induced ATP depletion, suggesting that treatment with insulin can maintain ATP despite impaired mitochondrial function. In addition, measurements of NAD(P)H autofluorescence revealed that insulin appeared to switch the mitochondrial metabolism towards a greater glycolytic contribution presumably sufficient to maintain ATP. Insulin also markedly potentiated the inhibition of the PMCA by glycolytic inhibitors, yet abolished inhibition of the PMCA by mitochondrial inhibitors. This further suggests that this insulin-induced metabolic switch towards glycolysis is not only sufficient to maintain ATP, but also makes the PMCA almost entirely dependent on glycolysis as the major ATP fuel. Therefore, even in the face of impaired mitochondrial function (e.g. following induction of pancreatitis by bile acids and fatty acid ethyl esters), insulin might be expected to enhance glycolytic ATP supply and thus maintain PMCA activity. This would help to prevent the cytotoxic Ca$^{2+}$ overload and the consequent spiral of self-perpetuating tissue damage which occurs during pancreatitis.

As outlined in the introduction, there is substantial evidence from several unrelated experimental animal models of pancreatitis that insulin (10-13) and other growth factors/peptides (17-19) are protective. There is also a body of indirect circumstantial clinical evidence in humans that insulin offers some protection against pancreatitis, for example by reducing the mortality rate and reducing symptomatic pain (14-16). However, data from the current study provides the first direct evidence that such protection occurs at the level of pancreatic acinar cells, a mechanism which could be exploited for the treatment of the disease.

Perhaps the simplest explanation for the protective effects of insulin is that insulin reduces intracellular oxidative stress. However, in the present study insulin had no significant effect on H$_2$O$_2$-induced oxidative stress, as measured by DCF fluorescence, suggesting that increased cellular antioxidant capacity is unlikely to be responsible for the insulin protection.

Insulin activates a number of different signalling pathways, but perhaps the most widely studied is the activation of phosphoinositide-3 kinase (PI-3K), the enzyme that converts PIP$_2$ to PIP$_3$. PIP$_3$ recruits Akt to the plasma membrane where it becomes activated by phosphorylation by phosphoinositide-dependent kinase (PDK-1) (21). There is a body of evidence in a wide variety of cells and tissues that activation of Akt-dependent signalling pathways is largely cytoprotective (21). Nevertheless, and at apparent variance with this cytoprotective action of insulin and/or PI3K/Akt, there is also strong evidence that activation of the PI3K pathways can promote pancreatic damage in several unrelated experimental models of pancreatitis (34-37). Notably, pancreatic injury and clinical markers of pancreatitis were markedly reduced in mice in which the catalytic subunit (p110) of PI3K$\gamma$ was deleted (p110$^-\gamma^-\$ mice) (35,36). Moreover, the PI3K inhibitor LY294002 attenuated the bile-acid-induced Ca$^{2+}$ overload responses and inhibition of SERCA activity in isolated pancreatic acinar cells, suggesting that activation of the PI3K pathway was responsible for these bile acid effects (34,38). However, the PI3K p110$\gamma$ isoform, implicated in the above studies, has been suggested to facilitate inflammation (39) and is classically activated by G-protein-coupled receptors (e.g. CCK in caerulein pancreatitis), rather than by tyrosine kinase receptors that are activated by insulin (40). Furthermore, Akt is not thought to be the major downstream effector of PI3K p110$\gamma$ (40). Low “physiological” concentrations of CCK (0.1 nM) have been shown to cause Akt phosphorylation in isolated pancreatic acinar cells, consistent with cytoprotection (41). This is supported by our previous study where H$_2$O$_2$ induced an irreversible Ca$^{2+}$ overload in fewer acinar cells when the cells were treated with 20 pM CCK (6). However, high “cytotoxic” CCK concentrations (10-1000 nM) reduced Akt phosphorylation to below basal levels (41), consistent with inhibition of Akt, which is more likely to exacerbate acinar cell injury. In contrast to stimuli acting through GPCRs, insulin activates the class 1A PI3K $p110\alpha$ isoform (42). This PI3K isoform classically couples to the activation of Akt and caused marked Akt phosphorylation in pancreatic acinar cells (41) consistent with our current study. Notably, one of the earliest studies to implicate PI3K in pancreatitis suggested that caerulein activates class III PI3K, leading to an increase in phosphotidylinositol 3-phosphate (PtdIns3P), perturbation of golgi stack-lysosome fusion, and the consequent intra-acinar trypsinogen activation (37). Therefore, we suggest that activation of different PI3K isoforms is likely to regulate diverse pathophysiological responses in pancreatic acinar cells, although activation of Akt is most likely to be cytoprotective.

The molecular mechanism for the protective effects of insulin, and in particular the activation of PI3K/Akt pathways, on Ca$^{2+}$ overload and inhibition of the PMCA in pancreatic acinar cells is likely to be complex, since PI3K/Akt couples to many...
downstream signalling pathways. These include increased cellular metabolism, cell proliferation, anti-apoptotic and pro-survival pathways (21). However, since the protective effects of insulin we observed were relatively short-term (15-30 minutes), they are unlikely to be due to increased transcription or expression, but rather suggest a rapid effect of post-translational signalling pathways, for example due to Akt phosphorylation.

Our data clearly demonstrate that insulin protects against oxidant-induced inhibition of the PMCA activity. Interestingly, insulin and IGF-1 have been reported to increase PMCA activity in kidney proximal tubule basolateral membranes (43). This insulin-induced increase in PMCA activity was abolished in streptozocin-induced diabetic rats (43) obese fa/fa rats (44) and ob/ob mice (45), most likely due to insulin resistance and a loss of insulin effectiveness. Similarly, brain synaptic PMCA activity has also been reported to be reduced in streptozocin-induced diabetic rats (46) an effect reversed by exogenously applied insulin (47). These data suggest that insulin, either endogenously released or exogenously applied, increases PMCA activity. However, our data showed that insulin had no direct effect on PMCA activity when applied alone (without H2O2) during the in situ [Ca2+]i clearance assay, or when applied to resting cells. The protective effects of insulin on PMCA activity in the present study thus appeared to be due to a reduction in oxidant-induced inhibition rather than a direct activation of the PMCA.

In our previous study H2O2-induced inhibition of the PMCA was independent of mitochondrial Ca2+ handling, but coincided with mitochondrial depolarisation and was attenuated by inhibitors of the mitochondrial permeability transition pore (mPTP), such as cyclosporine-A and bongrekic acid (5). This led us to hypothesise that inhibition of the PMCA was due in part to H2O2-induced opening of mPTP and mitochondrial depolarisation. Based on these observations it might be hypothesised that insulin protected the PMCA by reducing the opening of the mPTP and preventing mitochondrial depolarisation. Indeed, some of the major targets of Akt reside at or within the mitochondria, including the Bad-Bcl-2/Bcl-Xs complex and the voltage-dependent anion channel (VDAC)-hexokinase II (HK-II) complex (26-28). Both these pathways regulate the mPTP and facilitate the complex reciprocal regulation of metabolism and pro-survival pathways. However, to our surprise insulin had no effect on H2O2-induced mitochondrial depolarisation in the current study, suggesting that the mPTP plays no role in the protective effects of insulin.

Also in our previous study (5), low concentrations of H2O2 (50 µM) had no detectable effect on ATP depletion (measured with MgGreen) yet caused mitochondrial depolarisation and substantial inhibition of the PMCA. These data led us to conclude that inhibition of the PMCA appeared to be independent of ATP depletion—or more accurately, could occur prior to substantial ATP depletion—but rather was more dependent on mitochondrial membrane potential. We therefore speculated that perhaps this apparent “ATP depletion-independent” inhibition of the PMCA could be due to the release of some putative PMCA-inhibitory factor from the mitochondria, though, to date we have no direct evidence for such a mechanism. It is also important to note that part of the H2O2-induced inhibition of the PMCA may be due to direct oxidation of either the PMCA and/or calmodulin, which would occur independently of any mitochondrial depolarisation or ATP depletion.

In the present study, using the luminescence-based kit, H2O2 caused substantial ATP depletion over a narrow range of concentrations (30-300 µM). This appears to be contradictory to MgGreen experiments in which lower concentration had no effect (5) and 500 µM caused only 50% ATP depletion. This apparent discrepancy could be explained by the fact that the MgGreen technique selects for the very best cells likely to be the most resistant to ATP depletion within a cell population. The ATP kit, on the other hand, measures total ATP depletion across the entire population and therefore does not exclude those cells that are already dying and thus likely highly sensitive to ATP depletion.

Nevertheless, these new data re-instates ATP depletion as a mechanism potentially contributing to PMCA inhibition. More importantly, since insulin had no effect on oxidative stress or mitochondrial depolarisation, protection of the PMCA by insulin is likely to be due to prevention of ATP depletion. In addition, the residual, insulin-insensitive inhibition of the PMCA was likely independent of ATP depletion and more likely due to direct oxidation (or even the release of some putative mitochondrial PMCA inhibitory factor via the opening of the mPTP).

In the current study, the changes in NADH autofluorescence suggest that insulin “switches” cellular metabolism from predominantly mitochondrial to glycolytic metabolism which likely serves to maintain ATP supply to the PMCA in the face of oxidant-impaired mitochondrial function. In fact insulin treatment caused the PMCA to become exquisitely sensitive to glycolytic inhibitors, suggesting that insulin caused the PMCA to become
almost entirely dependent on glycolysis as an ATP supply. Such a metabolic switch, referred to as the “Warburg effect” after its discovery by Otto Warburg almost 90 years ago, is well documented to occur during cancer (48). In particular, PI3K and Akt are well documented to be constitutively up-regulated in cancer and have been suggested to play a major role in contributing to the metabolic phenotype of cancer cells (49). However, this Warburg effect is due to altered expression of metabolic enzymes and/or the signalling pathways that control these metabolic pathways (50), and thus unlikely to explain the acute insulin-induced “metabolic switch” observed over the short time frame of our NAD(P)H experiments. Nonetheless, PI3K/Akt is one of the major downstream signalling pathways activated by insulin, therefore it is reasonable to suggest that the acute effects of insulin treatment can lead to the post-translation regulation of metabolism and thus mimic a short-term metabolic phenotype of cancer cells.

There is substantial evidence that insulin, and activation of PI3K/Akt, increases glycolytic flux by activation of several steps of the glycolytic pathway. In particular, glycolytic flux is primarily regulated by the activity of phosphofructokinase-1 (PFK1), which catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate and represents the first irreversible and committed step in glycolysis. Insulin has been shown to directly activate phosphofructokinase-2 (PFK2) via Akt-mediated phosphorylation, which catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, the most potent allosteric activator of PFK1 (51). Despite this, evidence suggests that insulin, and activation of PI3K/Akt, can increase both mitochondrial oxidative phosphorylation and glycolytic metabolism (27). This is difficult to reconcile the insulin-induced decrease in “mitochondrial” NAD(P)H observed in the current study. Nevertheless, studies have shown that Akt can increase NAD(P)H, lactate and glucose consumption without affecting mitochondrial oxidative phosphorylation (52). Moreover, due to its anabolic nature, insulin not only increases glucose uptake and glycolytic flux but can also divert metabolic flux away from mitochondrial oxidative phosphorylation and towards fatty acid synthesis, glycogen synthesis, the pentose phosphate pathway and acetyl Co-A carboxylase, important regulatory steps that drive citrate from the Krebs cycle towards fatty acid synthesis (53-55). Collectively these studies are broadly in line with our observation that insulin reduces the relative “mitochondrial” NAD(P)H and increases “glycolytic” NAD(P)H.

The notion that insulin switches metabolism from predominantly mitochondrial to glycolytic metabolism may be extremely relevant in light of the published evidence that the PMCA has its own glycolytic ATP supply that may render it largely insensitive to inhibition of mitochondrial metabolism (56-58). Specifically, in isolated inside-out plasma membrane vesicles from pig stomach smooth muscle enriched with PMCA, an endogenous membrane-bound glycolytic system provided ATP to fuel the PMCA-dependent Ca2+ uptake (56,57). Moreover, providing glycolytic substrates were present, the Ca2+ uptake (PMCA activity) persisted in the absence of an exogenously applied ATP regenerating system (56,57). These studies suggest that key glycolytic enzymes may provide the PMCA with a “privileged” source of ATP to fuel the PMCA. Under physiological conditions, when the bulk cytosolic ATP concentration is saturating for the PMCA (i.e.> 1 mM), such close functional coupling between glycolytic enzymes and the PMCA is likely to be of minimal functional significance. In other words, the PMCA does not care where the ATP comes from; a mitochondrial source or glycolytic source. In fact it is likely that the majority of cytosolic ATP will come from mitochondria, rather than glycolysis. However, in the face of impaired mitochondrial metabolism, for example under conditions of cellular stress, perhaps a glycolytic source of ATP—or more specifically an insulin-mediated “up-regulated” glycolytic source of ATP—is likely to be critical in maintaining PMCA activity and thus restoring low resting cytosolic [Ca2+]. Under these stressed conditions, such a “privileged” source of ATP may be sufficient to “fuel” the PMCA even if bulk (global) ATP is close to zero.

Collectively these data suggest insulin protects the PMCA from oxidant-induced inhibition and the consequent Ca2+ overload by effectively redirecting ATP production from mitochondrial metabolism to glycolytic metabolism, which then fuels the PMCA in the face of impaired mitochondrial function. Such a mechanism may be very important in preventing pancreatic acinar cells from undergoing necrotic cell death during severe metabolic stress, such as when exposed to agents that induce pancreatitis. As such, we suggest this mechanism is potentially of considerable pathophysiological importance.
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ABBREVIATIONS

[Ca^{2+}], intracellular Ca^{2+} concentration; PMCA, plasma membrane Ca^{2+}-ATPase; H_{2}O_{2}, hydrogen peroxide; NCX, Na^{+}-Ca^{2+} exchange; PI3K, phosphoinositide-3 kinase; NADH, nicotinamide adenine dinucleotide; DCF, dichlorofluorescein; mitochondrial membrane potential (ΔΨm); TMRM, tetramethylrhodamine membrane methylster; CCCP, carbonylcyanide m-chlorophenylhydrazone; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; AUC, area under the curve; CPA, cyclophosphamide acid; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} entry; mPTP, mitochondrial permeability transition pore; IOA, iodoacetate; BrPyr, 3-bromopyruvate; CCK, cholecystokinin; PIP_{2}, phosphatidylinositol (4,5)-bisphosphate; PIP_{3}, phosphatidylinositol (3,4,5)-trisphosphate; PDK_{1}, phosphoinositide-dependent kinase; PFK1, phosphofructokinase-1; ACL, ATP-citrate lyase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethoxy ether)-N,N,N′,N′-tetraacetic acid; BSA, bovine serum albumin; CCD, charged coupled device.

FIGURE LEGENDS

FIGURE 1. Insulin protects the H_{2}O_{2}-induced Ca^{2+} overload in a PI3K-dependent manner. Fura-2-loaded pancreatic acinar cells were treated with 100 μM H_{2}O_{2} for 10 minutes where indicated. Cells were either untreated (control, n=15; A.) or pre-treated with 100 nM Insulin (Insulin, n=9; B.) or a combination of insulin and the PI3K inhibitor, LY294002 (10 μM) (Insulin + LY294002, n=15; C.) for 30 minutes prior to the addition of H_{2}O_{2}. Mean data (+ SEM) for maximum H_{2}O_{2}-induced change in resting [Ca^{2+}], (Max ΔCa^{2+};
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**FIGURE 2. Insulin has no effect on H₂O₂-induced oxidative stress** Representative traces showing the relative DCF fluorescence (F/F₀) in response to 500 µM H₂O₂ in untreated control pancreatic acinar cells (A) or following pre-treatment with 100 nM insulin for 30 minutes (B). **C.** Overall mean data (± SEM), showing the change in DCF fluorescence (%ΔF/F₀) in response to 0.1 (n=4), 0.5 (n=6) and 1 mM H₂O₂ (n=8) in the absence (light grey box) or presence (dark grey box) of 100 nM insulin. *p<0.05 represents statistical significance, p<0.05 by comparing to time-matched control experiments (white box) as determined by a non-parametric Mann Whitney U test. NS represents non-significance when compared between control and insulin-treated cells.

**FIGURE 3. H₂O₂ causes a concentration-dependent inhibition of PMCA activity in an in situ [Ca²⁺]i clearance assay.** Representative traces showing the in situ [Ca²⁺], clearance assay (see RESULTS) in untreated fura-2-loaded pancreatic acinar cells (time-matched control, n=8; A.) and cells treated with 50 µM H₂O₂ (n=4, B.), 100 µM H₂O₂ (n=6, C.), 500 µM H₂O₂ (n=6, D.), during the second influx-clearance challenge. Cells were treated with 30 µM CPA (arrow) in the absence of external Ca²⁺ with 1 mM EGTA (white box) or 20 mM Ca²⁺ (grey box) to induce the Ca²⁺ influx phase. **E.** shows expanded time-courses taken from the second clearance phase in A-D in the presence of increasing concentrations of H₂O₂. Linear clearance rate (in the presence of H₂O₂) was normalised to the initial clearance rate in each cell (% relative clearance). **F.,** Mean % relative clearance (± SEM), *p<0.05, **p<0.01 and ***p<0.001, compared to time-matched control experiments (black box) as determined by a non-parametric Mann Whitney U test.

**FIGURE 4. Insulin protects the H₂O₂-induced inhibition of PMCA activity in a PI3K-dependent manner.** Representative traces showing the in situ [Ca²⁺]i clearance assay A-D. Pancreatic acinar cells were treated with 100 µM H₂O₂ during the second influx-clearance challenge in untreated control cells (n=6, A.), cells pre-treated with 100 nM insulin (n=6, B.) or a combination of insulin and LY294002 (10 µM) (n=5, C.) for 30 minutes. **D.** Expanded time-courses taken from traces in A-C comparing the initial control clearance (black trace) with clearance during treatment with 100 µM H₂O₂ (grey traces), measured from the same standardised value (dotted line). **E.,** Mean data (± SEM) showing the effect of H₂O₂ on % relative clearance under the different treatment conditions. Statistical significance, *p<0.05, as determined by a non-parametric Mann Whitney U test.

**FIGURE 5. Insulin induces AktS473 phosphorylation in a PI3K-dependent manner.** Representative Western blots using the pAktS473 antibody and pan-Akt antibody on cell lysates from pancreatic acinar treated with (+) or without (-) 100 nM insulin and/or 10 µM LY294002 for 15 minutes (n=4). The pAktS473 antibody detects phosphorylated serine at position 473 on Akt and the pan-Akt antibody was used as a loading control.

**FIGURE 6. Insulin has no effect on H₂O₂-induced depolarisation of the mitochondrial membrane potential (ΔΨm).** Representative traces showing the relative TMRM fluorescence (F/F₀) of cells treated with 500 µM H₂O₂ alone (n=6, A), or in combination with 100 nM insulin (n=6, B) or the effect of 100 nM insulin alone (n=4, C). In all cases, (A-C), 4 µM CCCP was added to the cells to induce a maximum depolarisation as a positive control. **D.** Mean data were quantified and normalised by expressing the change in F/F₀ as a % of the CCCP response (*p<0.05, as assessed using a Mann Whitney test).

**FIGURE 7. Insulin attenuates H₂O₂-induced ATP depletion.** Cellular ATP was assessed using magnesium green (MgGreen) fluorescence and the in vitro ViaLight Plus luciferase-based chemiluminescence ATP monitoring kit. Representative traces showing the relative MgGreen fluorescence (F/F₀) in response to 500 µM H₂O₂ followed by the “ATP depletion” cocktail in untreated control cells (A) and cells pre-treated with 100 nM insulin for 30 minutes (B). The ATP depletion cocktail consisted of 100 µM CCh, 10 µM oligomycin and 2 mM iodoacetate, 500 µM bromopyruvate and 4 µM CCCP to induce maximum ATP depletion. **C.** Responses to H₂O₂ were quantified and normalised by expressing the change in F/F₀ as a % of the ATP depletion cocktail response and compared between untreated control cells and insulin-treated cells (*p<0.05, as assessed using a Mann Whitney U test). **D.** Average H₂O₂ concentration...
ATP depletion response curves for untreated control cells (filled squares) and cells pre-treated with 100 nM insulin (open circles), measured using the ATP kit. Pancreatic acinar cells were treated with or without 100 nM insulin for 20 minutes, followed by treatment with or without various concentrations of H₂O₂ (0-1 mM) for a further 20 minutes. Luminescence of the “ATP-depletion cocktail” (maximum ATP depletion) was subtracted prior to normalisation to the corresponding time-matched control (total ATP). Additional control experiments were performed in which cells were treated with 100 nM insulin alone, without H₂O₂ (open triangle).

FIGURE 8. Effect of insulin on the relative contributions of mitochondrial versus glycolytic NAD(P)H autofluorescence in pancreatic acinar cells. The relative contributions of mitochondrial versus glycolytic NAD(P)H autofluorescence was determined by treating with 4 µM CCCP, to deplete mitochondrial NAD(P)H, and 2 mM iodoacetate (IOA), to inhibit glycolytic NAD(P)H production. Representative traces of raw background-subtracted autofluorescence grey levels for untreated control cells (n=6, A) and cells pre-treated with 100 nM insulin (n=7, B). The relative CCCP and IAA-induced decrease in NAD(P)H autofluorescence was quantified and normalised by expressing as a percentage of the total decrease in NAD(P)H autofluorescence. C, Mean data (± SEM) for control and insulin-treated cells (*p<0.05, as assessed using a Mann Whitney test).

FIGURE 9. Insulin potentiates BrPyr-induced inhibition of the PMCA and abolishes the CCCP-induced inhibition of the PMCA. Representative traces showing the in situ [Ca²⁺]i clearance assay A–D, following treatment with metabolic inhibitors. Pancreatic acinar cells were treated with 500 µM 3-bromopyruvate (BrPyr; A and B; n=7 and 8 respectively) or CCCP (C and D; n=8 and 8 respectively) during the second influx-clearance challenge in untreated control cells (A and C) or cells pre-treated with 100 nM insulin for 30 minutes (B and D). Expanded time-courses taken from each trace in A–D are shown in the adjacent inset comparing the initial control clearance (black trace) with clearance during metabolic inhibitor (grey trace) superimposed and measured from the same standardised value (dotted line). E, Mean data (± SEM) showing the effect of each metabolic inhibitor on % relative clearance in the absence and presence of insulin. The white box represents the time-matched controls, the black and the grey boxes represent the effect of metabolic inhibitors on mean normalised Ca²⁺ clearance in untreated control cells and insulin-treated cells respectively. Statistical significance, *p<0.05, as determined by a non-parametric Mann Whitney U test, compared to time-matched control and **p<0.05, comparing between groups.
Figure 3

A. Time-matched control

B. 50µM H₂O₂

C. 100µM H₂O₂

D. 500µM H₂O₂

E. Normalised Rate (% R₂/R₁)

F. [H₂O₂] (µM)
Figure 5

| Kd | — | — | — | + | + | — | — | + | + | + | + |

LY294002 (10 μM)
Insulin (100 nM)

pAktS473
Akt
Figure 7

A  Control

Mg Green
$\Delta F/F_0$

$500 \mu M \text{ H}_2\text{O}_2$

10 min

B  Insulin

Mg Green
$\Delta F/F_0$

$500 \mu M \text{ H}_2\text{O}_2$

10 min

C  Mg Green fluorescence

D  Luciferase luminescence (ATP kit)

% of ATP depletion cocktail

% Control

$\text{H}_2\text{O}_2$

$\text{H}_2\text{O}_2$

Insulin

$[\text{H}_2\text{O}_2]$ (µM)

Insulin alone
**Figure 8**

**A** NAD(P)H Autofluorescence

*Control*

- 4 μM CCCP
- 2 mM IOA

**B** NAD(P)H Autofluorescence

*Insulin*

- 4 μM CCCP
- 2 mM IOA

**C**

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*Relative NAD(P)H (% Total)*

- * indicates significant difference.
Insulin protects pancreatic acinar cells from cytosolic calcium overload and inhibition of the plasma membrane calcium pump

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