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α-Lipoic acid regulates AMP-activated protein kinase and inhibits insulin secretion from beta cells

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Abstract *Aims/hypothesis:* The antioxidant compound α-lipoic acid (α-LA) possesses antidiabetic and anti-obesity properties. In the hypothalamus, α-LA suppresses appetite and prevents obesity by inhibiting AMP-activated protein kinase (AMPK). Given the therapeutic potential of α-LA for the treatment of type 2 diabetes and obesity, and the importance of AMPK in beta cells, we examined the effect of α-LA on pancreatic beta cell function. *Materials and methods:* Isolated rat islets and MIN6 beta cells were treated acutely (15–90 min) or chronically (18–24 h) with α-LA or the known AMPK-activating compounds 5′-amino-imidazole-4-carboxamide ribonucleoside (AICAR) and metformin. Insulin secretion, the AMPK-signalling pathway, mitochondrial function and cell growth were assessed. *Results:* Acute or chronic treatment of islets and MIN6 cells with α-LA led to dose-dependent rises in phosphorylation of the AMPK α-subunit and acetyl CoA carboxylase. Chronic exposure to α-LA, AICAR or metformin caused a reduction in insulin secretion. α-LA inhibited the p70 s6 kinase translational control pathway, and inhibited MIN6 growth in a manner similar to rapamycin. Unlike AICAR and metformin, α-LA also acutely inhibited insulin secretion. Examination of the effect of α-LA on mitochondrial function showed that acute treatment with this compound elevated reactive oxygen species (ROS) production and enhanced mitochondrial depolarisation induced by Ca^{2+}. *Conclusions/interpretation:* This study is the first to demonstrate that α-LA directly affects beta cell function. The chronic effects of α-LA include AMPK activation and reductions in insulin secretion and content, and cell growth. Acutely, α-LA also inhibits insulin secretion, an effect probably involving the ROS-induced impairment of mitochondrial function.

Keywords AMPK · Insulin secretion · α-Lipoic acid · Mitochondrial permeability transition · mTOR · Pancreatic beta cell · Reactive oxygen species

Abbreviations α-LA: α-lipoic acid · $\Delta\Psi_m$: Mitochondrial membrane potential · ACC: acetyl CoA carboxylase · AICAR: 5′-amino-imidazole-4-carboxamide ribonucleoside · AMPK: AMP-activated protein kinase · DCF: dichlorofluorescein · GSIS: glucose-stimulated insulin secretion · KRBH: Krebs–Ringer buffer with HEPES · MPT: mitochondrial permeability transition · mTOR: mammalian target of rapamycin · p70 s6k: p70 s6 kinase · ROS: reactive oxygen species

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Introduction

α-Lipoic acid (α-LA) is a short-chain fatty acid that acts as a cofactor of enzymes involved in mitochondrial respiration [1]. Beneficial anti-oxidative properties for α-LA have been described in several tissues, including the brain [2], kidney [3] and heart [4]. In recent years, evidence has accumulated suggesting that α-LA has a wide range of benefits in the treatment of diabetes. Bitar et al. [5] demonstrated that α-LA could partly ameliorate insulin resistance in type 2 diabetic rats, while others had reported additional antidiabetic effects in earlier rodent [4, 6], and human studies [7]. In vitro approaches have also shown that α-LA can enhance glucose disposal in skeletal muscle [8] and adipocytes [9], and suppress hepatic gluconeogen-

esis [10]. The exact molecular mechanisms responsible for these effects are currently not known.

AMP-activated protein kinase (AMPK) functions as an energy sensor in mammalian cells (reviewed in [11]). AMPK is a heterotrimeric enzyme that is activated by stress signals [12], such as an elevated intracellular AMP: ATP ratio. When AMP levels rise, the AMPK α-subunit becomes phosphorylated and activated, leading to the suppression of anabolic processes, including protein and lipid synthesis. This is accompanied by an increase in ATP-generating pathways, including the β-oxidation of fatty acids. AMPK mediates insulin-independent glucose uptake in skeletal muscle [13], suppresses glucose production from the liver [14], and is activated by the hormones leptin [15] and adiponectin [16], and the antidiabetic drug metformin [17]. As such, AMPK has attracted much attention as a potential target for diabetes therapy.

Recently, the properties of α-LA were linked to AMPK in a report describing its ability to prevent obesity in rats [18]. α-LA treatment reduced body weight and feeding and increased energy expenditure in these rodents. These effects were shown to be, at least in part, due to the ability of α-LA to suppress AMPK activity in the hypothalamus. Injection of rats with 5′-amino-imidazole-4-carboxamide ribonucleoside (AICAR), an AMP analogue and potent activator of AMPK, reversed the effects of α-LA and prevented protection from obesity.

In pancreatic islets of Langerhans, AMPK possesses a unique role, connecting cellular energy status to the capacity of beta cells to synthesise and secrete insulin. A number of studies have demonstrated that AMPK, when activated, transmits the signal of low glucose availability and leads to an inhibition of insulin secretion and expression [19–22]. While a role for AMPK in the beta cell continues to unfold, evidence for a role for α-LA in islet function is lacking. Given that α-LA modulates AMPK activity in the hypothalamus, and AMPK regulates insulin secretion, we investigated the possible role of α-LA in beta cell function using isolated rat islets and the MIN6 beta cell line.

Materials and methods

Animals

Male C57/Bl6 mice, 2 to 3 months of age, were purchased from Charles River (Wilmington, MA, USA). All animals were handled according to the guidelines of the Canadian Council on Animal Care. All the protocols and procedures were approved by the Animal Care and Use Committee at the University of Toronto.

Reagents

α-LA and metformin (1,1-dimethylbiguanide hydrochloride) were obtained from Sigma (Oakville, ON, Canada). AICAR was from Toronto Research Chemicals (North York, ON, Canada). Rapamycin and all primary antibodies except anti-p70 s6 kinase (p70 s6k; Upstate, Lake Placid, NY, USA) were from Cell Signaling Technology (Beverly, MA, USA). Materials for rat insulin RIA were obtained from Linco Research (St Charles, MO, USA).

Cell culture

MIN6 insulinoma cells (passage 38–52), a gift from S. Seino (Chiba University, Chuo-ku, Japan), were cultured in Dulbecco's modified Eagle's medium with 25 mmol/l glucose, 10% FBS, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulphate, and β-mercaptoethanol (1.7 μl/500 ml) at 37°C and 5% CO_2. For overnight treatments, cells were cultured in growth medium with α-LA, metformin or AICAR at the specified concentrations. For acute treatment for AMPK immunoblots, MIN6 cells were washed twice in KRBH buffer (128.8 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l NaH_2CO_3, and 10 mmol/l HEPES, pH 7.4 with 0.1% BSA), 2.8 or 15 mmol/l glucose, and then incubated in the same medium for 30 min or 1 h. Cells were then washed twice before treatment with α-LA.

Islet isolations

Islets of Langerhans were isolated from male Wistar rats by collagenase digestion and separated by density gradient centrifugation. Islets were maintained in RPMI containing 11.1 mmol/l glucose, 10 mmol/l HEPES, 10% FBS, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulphate and cultured at 37°C and 5% CO_2.

Immunoblotting

Samples were separated by SDS-PAGE on 8 to 12% polyacrylamide gels and transferred to PVDF-Plus membranes (BIORAD). Primary antibodies (see above) were detected with donkey anti-rabbit or anti-mouse (Amersham Biosciences, Baie d'Urfe, QC, Canada) at 1:7,500 for 1.5 h at room temperature. Visualisation was by chemiluminescence (ECL or ECL Plus; Amersham Biosciences) and exposure to Kodak film (Eastman Kodak, Rochester, NY, USA). Phospho-blots were normalised by stripping membranes and re-probing with polyclonal antibodies to AMPK, Akt or p70 s6k to control for loading. Band densities were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA).

Insulin secretion

MIN6 cells were seeded in a 24-well plate at a density of ~2×10^5 per well. Twenty-four hours later, cells were treated with the indicated reagents for overnight incuba-

tion. Cells were then washed in glucose-free KRBH three times and pre-incubated twice for 30 min with the same medium. Following two more washes in KRBH, cells were incubated for 1 h in KRBH containing either 0 or 15 mmol/l glucose as previously described [23]. Medium was saved and assayed for insulin content by RIA. For experiments involving acute treatment of MIN6 cells with α-LA, AICAR or metformin, the respective compounds were added during the 1-h secretion period. All values were normalised to total DNA content.

Freshly isolated rat islets were cultured overnight and transferred to 6- or 12-well plates (20–25 islets per well) containing medium with the indicated treatments. Following overnight incubation, islets were washed in KRBH containing 2.8 mmol/l glucose, pre-incubated for 30 min in the same medium and then transferred to KRBH (2.8 mmol/l glucose) for 90 min, followed by 90 min in KRBH with 16.7 mmol/l glucose. Medium was collected for insulin RIA. For acute treatments, α-LA was included during the 90-min secretion stages, and islets were used 24 h after isolation. Islets were lysed in 70% acid-ethanol solution for DNA quantification and subsequent normalisation.

Perfusions

Surgery and pancreatic perfusions were performed as described [24] on male C57/Bl6 mice, with minor modifications. KRBH medium was the same as for experiments in isolated islets and MIN6 cells except for the addition of 3% dextran and a BSA concentration of 0.25%. Samples were collected after a 20-min pre-perfusion period.

Cell growth

Cell growth was assessed using the XTT cell proliferation assay (Proliferation Kit II; Roche Applied Sciences, Laval, QC, Canada) according to the manufacturer's instructions. Briefly, 2.5×10^4 cells per well were seeded into a 96-well tissue culture plate. Twenty-four hours after seeding, cells were treated with either 2.0 mmol/l α-LA or 5 nmol/l rapamycin and cultured for 24 to 72 h, with daily refreshing of medium. Absorbance readings at 490 nm were recorded following the indicated treatment and 6 h of incubation with the XTT reagent.

Microscopy

MIN6 cells were seeded onto coverslips, and treated for 24 h with either 2.0 mmol/l α-LA or 6 μmol/l staurosporine (Sigma). Cells were then incubated for 20 min with propidium iodide (Sigma) in medium containing (in mmol/l) 130 NaCl, 5 KCl, 5 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. Coverslips were washed and transferred to an open chamber with the same medium. Fluorescent imaging was carried out using an Olympus BX51W1 microscope fitted with a 20×/0.95 water immersion objective and cooled CCD camera at 36±1°C. For excitation, a xenon-lamp-based DeltaRam high-speed monochromator (Photon Technology International [PTI], London, ON, Canada) was used. Excitation and emission wavelengths were 540 and 660 nm, respectively. ImageMaster 3 software (PTI) was used for monochromator and videocamera control, as well as for imaging and data collection.

Mitochondrial membrane potential ($\Delta\Psi_m$), oxygen consumption and reactive oxygen species (ROS) production

Measurements were performed as previously described [25], except that α-LA was added to the cells prior to measurements. Oxygen consumption measurements were performed using a Clark-type electrode coupled to an Oxygraph unit (Hansatech, Pentney, UK). $\Delta\Psi_m$ was monitored by observing safranin O fluorescence using a FluoroCount plate reader (Packard Instruments, Meriden, CT, USA) at excitation/emission wavelengths of 530/590 nm. A decrease in fluorescence corresponded to an increase in $\Delta\Psi_m$. ROS production was assayed as hydrogen peroxide-induced formation of dichlorofluorescein (DCF; Sigma) from the non-fluorescent reduced form, and monitored at excitation/emission wavelengths 485/530 nm. DCF was obtained from the stable compound DCF diacetate by alkaline hydrolysis.

Statistics

Data are presented as means±SE. Significance between groups was determined using an unpaired two-tailed Student's *t*-test or one-way ANOVA when appropriate. Significance was established at $p<0.05$.

Results

Activation of AMPK signalling

Treatment of islets with 2.0 mmol/l α-LA (a concentration similar to that used in other in vitro studies [8, 9]) for 15 min led to an increase in phosphorylation of the AMPK α-subunit at Thr172 (2.54±0.14 fold) compared with untreated islets ($p<0.001$) (Fig. 1a,b). MIN6 cells treated with α-LA (0.5–2.0 mmol/l) for 30 min also exhibited an increase in AMPK α-subunit phosphorylation (Fig. 1c,d), a trend that occurred in a dose-dependent manner. The effect of α-LA on downstream signalling of AMPK was also detected, demonstrating that AMPK activity was increased. AMPK phosphorylates acetyl CoA carboxylase (ACC) at Ser79 leading to an inhibition of fatty acid synthesis and an increase in the rate of β-oxidation [15]. α-LA treatment of islets and MIN6 cells caused phosphorylation of ACC, a trend that increased dose-dependently in MIN6 cells

(Fig. 1). Figure 1e shows that AMPK is inhibited by glucose, and that α-LA activated AMPK at both low (2.8 mmol/l) and high (15 mmol/l) glucose concentrations.

We also investigated if α-LA caused a sustained increase in AMPK activity. AMPK phosphorylation in rat islets treated for 18 h with α-LA was increased by 1.58±0.24 fold compared with controls (Fig. 2a,b). MIN6 cells treated for 18 h with α-LA (0.5–2.0 mmol/l) also showed a sustained phosphorylation of AMPK (Fig. 2c,d). ACC phosphorylation was also detected in both MIN6 cells and islets following overnight treatment with α-LA.

Inhibition of insulin secretion

Activation of AMPK in beta cells using AICAR or metformin results in a reduced capacity for insulin secretion [21, 26, 27]. To investigate if α-LA would elicit similar effects, isolated rat islets were cultured for 18 h in 2.0 mmol/l α-LA or 1.0 mmol/l AICAR. The AMP analogue AICAR allosterically binds AMPK, making it a better target for phosphorylation and activation by an upstream kinase [28]. We observed an inhibition of glucose-stimulated insulin secretion (GSIS) at 16.7 mmol/l glucose by α-LA (63±5.8% of controls, $p<0.01$) and AICAR (64±6.9% of controls, $p<0.05$) (Fig. 3a). When the experiments were repeated with metformin (1.0 mmol/l), another AMPK-activating compound, we found that following an 18-h treatment, insulin

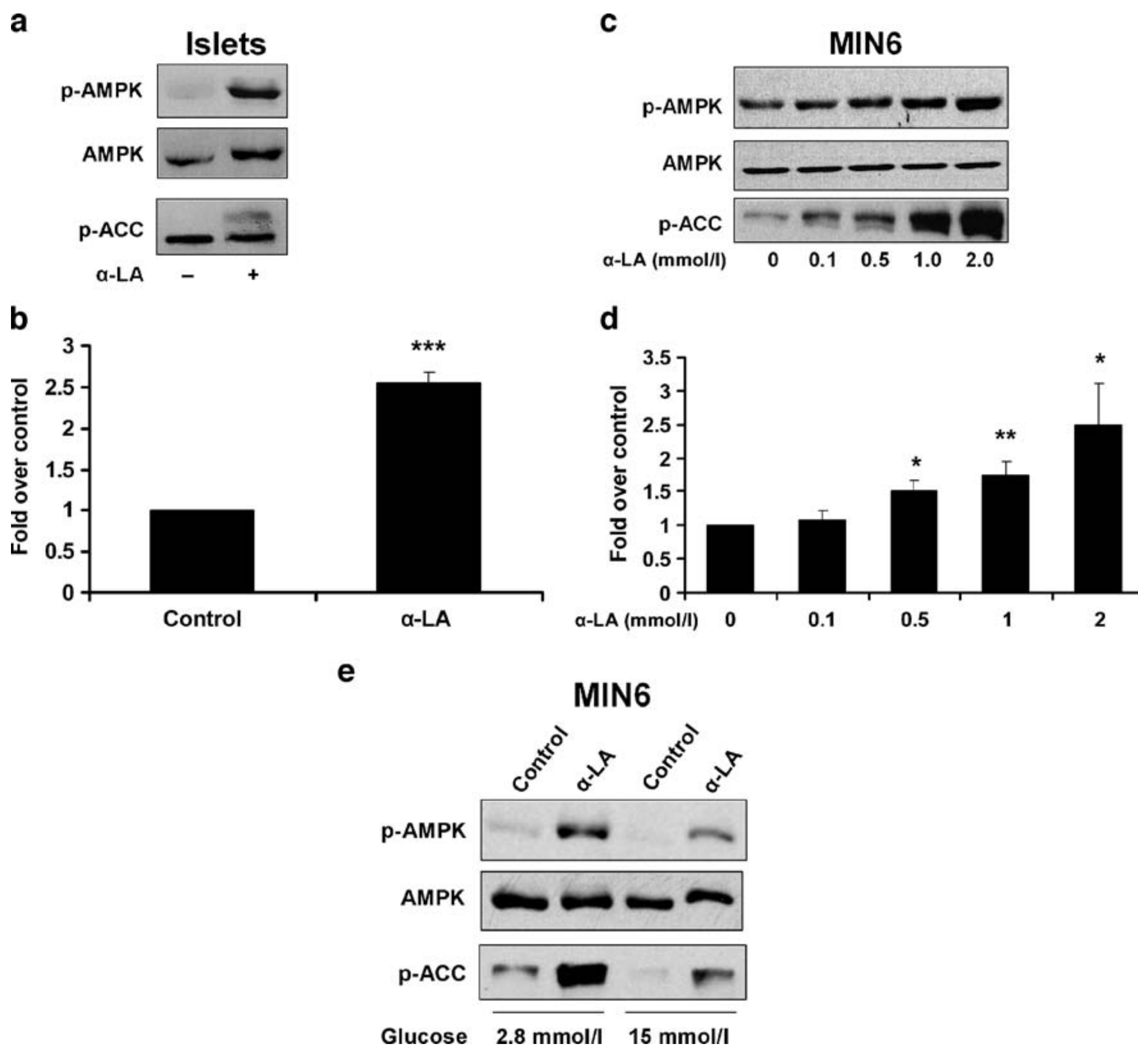

Fig. 1 α-LA acutely induces AMPK phosphorylation in rat islets and MIN6 cells. **a** Isolated rat islets were incubated in KRBH containing 2.8 mmol/l glucose for 1 h and then 16.7 mmol/l glucose with or without 2.0 mmol/l α-LA for 15 min. Representative immunoblots for phospho (p)- and total AMPK and phospho-ACC. **b** Relative changes in AMPK phosphorylation, represented as band density for phospho-AMPK normalised to total AMPK (*n*=3). **c** MIN6 cells were cultured for 30 min in KRBH containing the indicated concentrations of α-LA and glucose (15 mmol/l). Representative blots for phospho- and total AMPK and phospho-ACC. **d** Relative changes in AMPK phosphorylation (*n*=4). **e** MIN6 cells were treated for 1 h in KRBH containing 2.8 or 15 mmol/l glucose in the presence or absence of 2 mmol/l α-LA. Immunoblots for phospho- and total AMPK and phospho-ACC. Results are shown as means±SE. $*p<0.05$; $**p<0.01$; $***p<0.001$

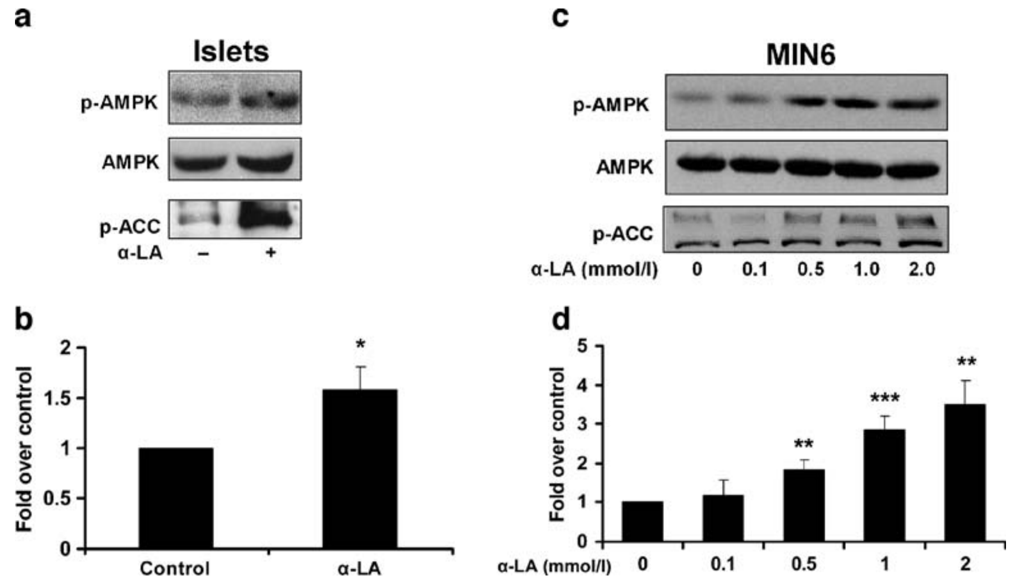

Fig. 2 Effect of chronic (18 h) α-LA treatment on AMPK in MIN6 cells and rat islets. **a** Isolated rat islets were cultured overnight in 2.0 mmol/l α-LA. Representative immunoblots are for phospho (p)- and total AMPK and phospho-ACC. **b** Band densities for phospho- and total AMPK (n=9). **c** MIN6 cells were cultured overnight in the indicated concentrations of α-LA. Representative immunoblots are for phospho- and total AMPK and phospho-ACC. **d** Relative changes in AMPK phosphorylation analysed by densitometry. Results are shown as means±SE, (n=3). *p<0.05; **p<0.01; ***p<0.001

secretion under high glucose (16.7 mmol/l) was 25±7.3% less than untreated control islets (p<0.05, data not shown).

Similar observations were made with MIN6 cells treated chronically with α-LA, AICAR or metformin. Compared with controls, α-LA (2.0 mmol/l) attenuated insulin secretion at 15 mmol/l glucose by 30±5.3% (p<0.01) (Fig. 3b). AICAR (1.0 mmol/l) and metformin (1.0 mmol/l) also reduced GSIS, by 55±4.2% (p<0.001) and 50±3.7% (p<0.001), respectively. At low glucose, α-LA inhibited secretion by 25±7.3% (p<0.05). As shown in Fig. 3c, immunoblots confirmed that AMPK activation was achieved with all three drugs. Total insulin content in MIN6 cells was significantly decreased in cells treated overnight with α-LA and AICAR (~70% of controls, p<0.001 for both conditions, Fig. 3d). In order to rule out non-specific effects of α-LA due to cytotoxicity, we

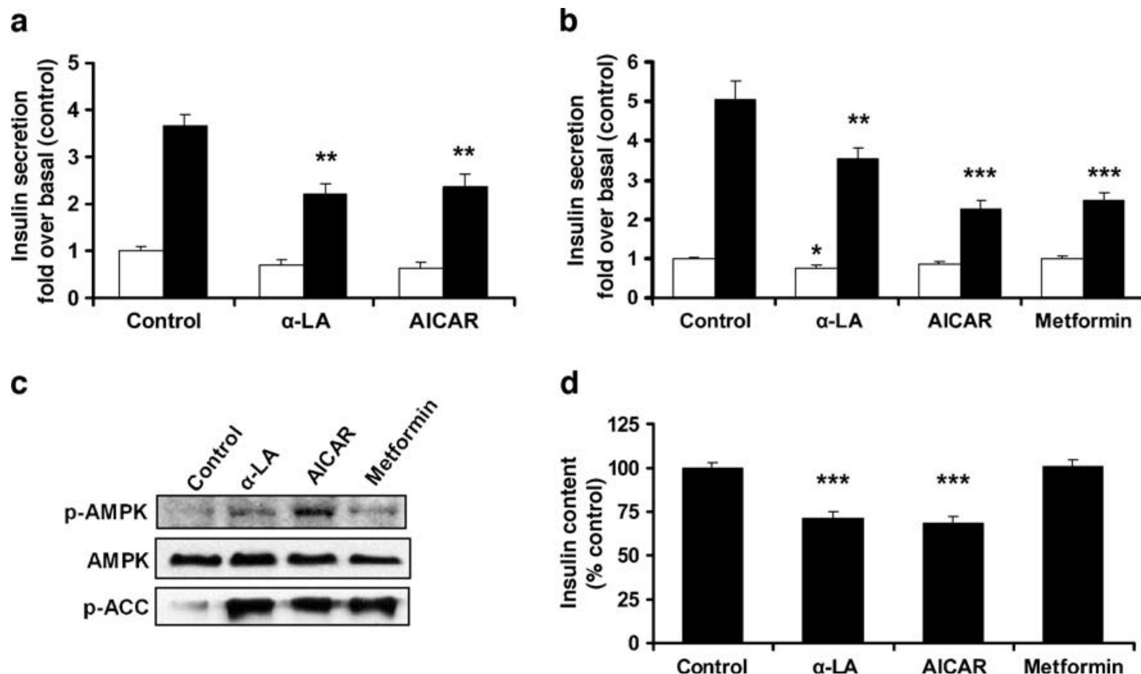

Fig. 3 Inhibition of insulin secretion by α-LA. **a** Isolated rat islets were cultured for 18 h in medium containing 2.0 mmol/l α-LA or 1.0 mmol/l AICAR and assayed for insulin secretion. Secretion was measured following 90 min incubation at 2.8 (*open bars*) or 16.7 mmol/l (*closed bars*) glucose (n=4). **b** MIN6 cells were cultured overnight in 2.0 mmol/l α-LA, 1.0 mmol/l AICAR or 1.0 mmol/l metformin. Insulin secretion was measured following 1 h in KRBH containing either 0 (*open bars*) or 15 mmol/l (*closed bars*) glucose (n=3–4). **c** Representative blots for phospho (p)-AMPK, total AMPK and phospho-ACC in MIN6 cells are shown for each condition following 18 h treatment as in (**b**). **d** Total insulin content was measured from MIN6 cells cultured overnight in 2.0 mmol/l α-LA, 1.0 mmol/l AICAR or 1.0 mmol/l metformin (n=3–4). All values were normalised to total DNA. *p<0.05; **p<0.01; ***p<0.001 compared with control values. Results are given as means±SE

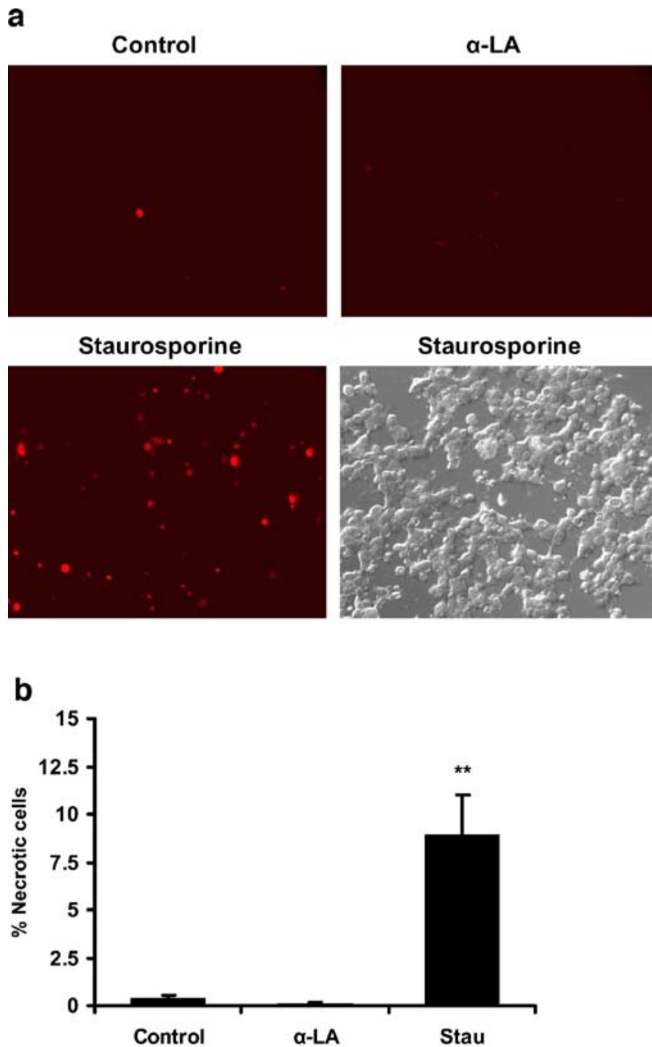

Fig. 4 Effect of α-LA on cell viability. Representative images (**a**) of MIN6 cells stained with propidium iodide following a 24-h treatment with control medium, 2.0 mmol/l α-LA or 6 μmol/l staurosporine (*Stau*). Light microscopy is also shown for staurosporine-treated cells. **b** The percentage of necrotic cells treated under the above conditions, with results given as the means±SE (n=3). **p<0.01

measured the effect of this compound on necrosis. Cell death in MIN6 cells treated with 2.0 mmol/l α-LA for 24 h was not different from controls, whereas staurosporine, an inducer of cell death [29], caused marked necrosis (Fig. 4).

Acute inhibition of insulin secretion

In addition to inhibiting secretion after overnight exposure, we found that α-LA also had acute inhibitory effects. When rat islets were challenged with glucose in the presence of α-LA, we observed a 45±3% (p<0.01) inhibition of GSIS compared with controls (Fig. 5a). Also, α-LA acutely activated AMPK, and inhibited GSIS from MIN6 cells at both 0 mmol/l (29±5.1%, p<0.01) and 15 mmol/l glucose (28±5.2%, p<0.05) compared with controls. Interestingly, despite inducing phosphorylation of AMPK, AICAR and metformin did not affect GSIS (Fig. 5b,c). Subsequent experiments in isolated rat islets acutely treated with AICAR and metformin also showed no significant effect (not shown). In addition to its effects on isolated islets and MIN6 cells during a static glucose challenge, α-LA (0.5 mmol/l) dramatically reduced GSIS in the perfused mouse pancreas (Fig. 5d). This effect was primarily evident in the first phase of insulin secretion (during the first 6–12 min).

Mammalian target of rapamycin (mTOR)/p70 s6k signalling and cell growth

Signalling by mTOR controls translation and cell growth (reviewed in [30]), and is inhibited by AMPK [31]. We found that α-LA reduced phosphorylation of the mTOR target p70 s6k at Thr389 by 63±7% (Fig. 6a,b). AICAR and metformin also inhibited p70 s6k, by 50±5 and 46±13%, respectively (Fig. 6a,b). As well, MIN6 cells treated for 24 h with α-LA (2 mmol/l), AICAR (1 mmol/l) or rapamycin (5 nmol/l), an inhibitor of mTOR, also had markedly reduced phosphorylation of p70 s6k (Fig. 6c). We also observed a decrease in the phosphorylation of the s6 ribosomal subunit (Ser235/236) and eukaryotic initiation factor 4E-binding protein 1 at Ser65 (Fig. 6d), two phosphorylation targets of mTOR/p70 s6k signalling [32, 33]. The phospho-state of Akt (Thr308) and eukaryotic initiation factor 4E (Ser209), other molecules involved in translational control, remained unchanged (Fig. 6d). As well, we found that cell growth was decreased similarly in MIN6 cells treated for 24–72 h with α-LA (Fig. 6e) or rapamycin (Fig. 6f), compared with controls, as indicated by an XTT cell proliferation assay.

Effect of rapamycin on insulin secretion

In order to determine if the decrease in insulin secretion and insulin content we observed with α-LA was due to an inhibition of mTOR, we investigated the effect of rapamycin on these parameters. Interestingly, MIN6 cells treated with 5 nmol/l rapamycin for 24 h actually secreted more insulin than untreated controls (129±13% at low glucose, 136±13% at high glucose, both p<0.05), possibly in part due to an increase in insulin content (121±8% compared with controls, p<0.01), as shown in Fig. 6g,h. These data suggest that while mTOR may mediate the effect of α-LA on cell growth, it is unlikely that it regulates the reduction in GSIS and total insulin content.

$\Delta\Psi_m$ and ROS production

The acute inhibitory effect of α-LA on insulin secretion appeared to have an AMPK-independent component. Given the reported ability of α-LA to induce a mitochondrial uncoupling effect [34], and that the primary regulator of insulin secretion, cytosolic ATP:ADP ratio, is main-

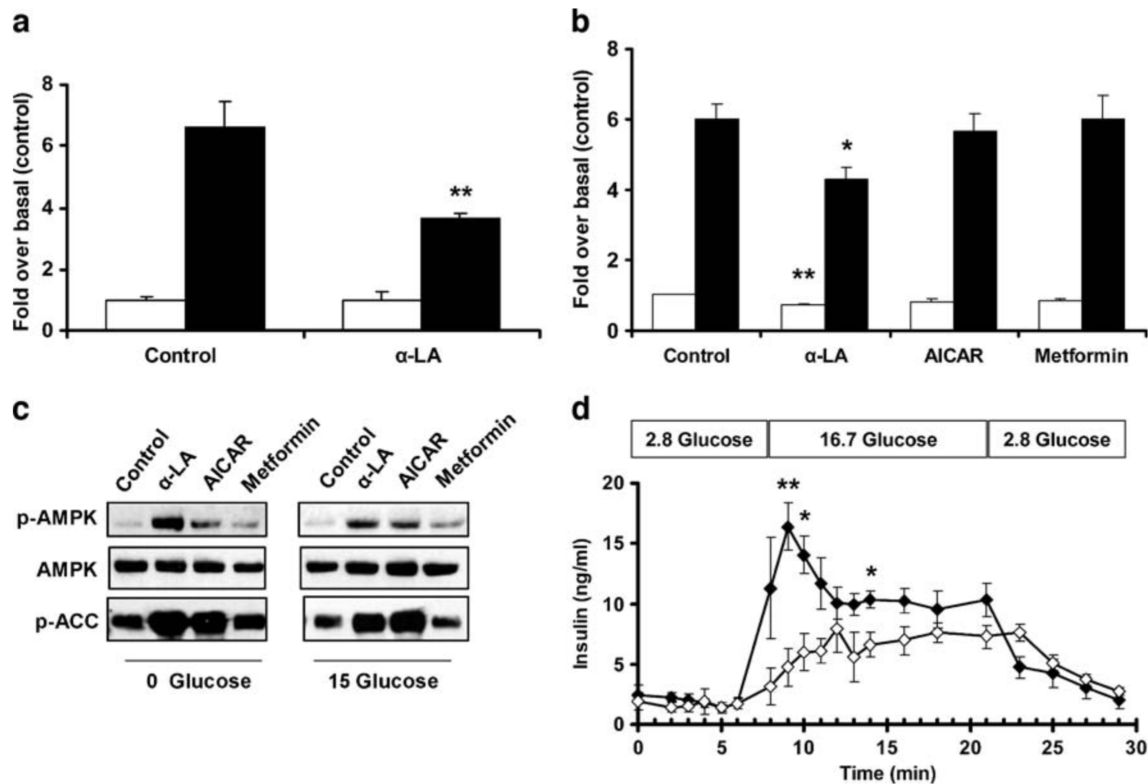

Fig. 5 Acute inhibition of insulin secretion by α-LA. **a** Rat islets were cultured for 90 min in KRBH with 2.8 mmol/l (*open bars*) or 16.7 mmol/l (*closed bars*) glucose in the absence/presence of 2.0 mmol/l α-LA (*n*=4). **b** MIN6 cells were incubated in 0 (*open bars*) or 15 mmol/l (*closed bars*) glucose in the presence of 2.0 mmol/l α-LA, 1.0 mmol/l AICAR or 1.0 mmol/l metformin for 1 h and assayed for insulin secretion at 0 or 15 mmol/l glucose (*n*=3). All results are means±SE. **c** Representative immunoblots for phospho (p)- and total AMPK and phospho-ACC are shown for each condition in (**b**). **d** Mouse pancreata were perfused with (*open symbols*) or without (*closed symbols*, control) KRBH containing 0.5 mmol/l α-LA. Glucose concentrations are shown in mmol/l. Samples were collected after a pre-perfusion period of 20 min at 2.8 mmol/l glucose. Data are given as means±SE from four separate animals under each condition. **p*<0.05; ***p*<0.01 compared with untreated controls

tained by mitochondrial oxidative phosphorylation [35], we tested the effect of this compound on mitochondrial function in permeabilised MIN6 cells. Oxidative phosphorylation was not affected by α-LA (200 μmol/l), but mitochondrial Ca^{2+} handling, which is another important mitochondrial function, was clearly dependent on α-LA. Incremental addition of Ca^{2+} to respiring mitochondria causes acceleration of oxygen consumption and membrane depolarisation due to Ca^{2+} uptake into the matrix [25], both of which were more pronounced in the presence of α-LA (Fig. 7a,b,d). Both parameters could be reverted to initial levels with cyclosporin A, a classic inhibitor of Ca^{2+}-induced mitochondrial permeabilisation (so-called mitochondrial permeability transition [MPT] [36]). MPT is an opening of non-specific pores in the mitochondrial inner membrane and is induced by Ca^{2+} in the presence of a variety of co-inducers, such as inorganic phosphate, thiol oxidants and fatty acids (reviewed in [36]). Next, we considered how α-LA might influence MPT opening. Since MPT can be induced by ROS [36], and α-LA can behave as both a pro-oxidant [34] and inducer of MPT [37], we tested the effect of α-LA on mitochondrial ROS production. Indeed, kinetics of respiration-dependent DCF oxidation (Fig. 7c,e), which reflects oxygen radical production in the respiratory chain, demonstrated increased mitochondrial ROS production in the presence of α-LA (200 μmol/l). Thus, our data suggest that α-LA can elevate the mitochondrial level of ROS and probability of MPT opening.

Discussion

In this report, we have demonstrated several novel properties of α-LA, including the activation of AMPK in rat islets and MIN6 cells. The role of α-LA as a regulator of AMPK has only recently been demonstrated. Whereas Kim et al. [18] showed that this compound inhibited hypothalamic AMPK activity, we have shown the opposite to be true in beta cells. Our observation of AMPK activation by α-LA in pancreatic beta cells suggests that AMPK is responsible for some of the reported effects found with α-LA in other tissues. For example, α-LA facilitates glucose uptake in both skeletal muscle and adipose tissue in vitro [8, 9]. Indeed, others have demonstrated that AMPK activation in these tissues leads to improved insulin sensitivity and enhanced glucose uptake and utilisation [13, 15–17]. Furthermore, both AMPK activation [14, 17] and α-LA [10] are associated with suppression of hepatic glucose output. Very recently, two reports described that

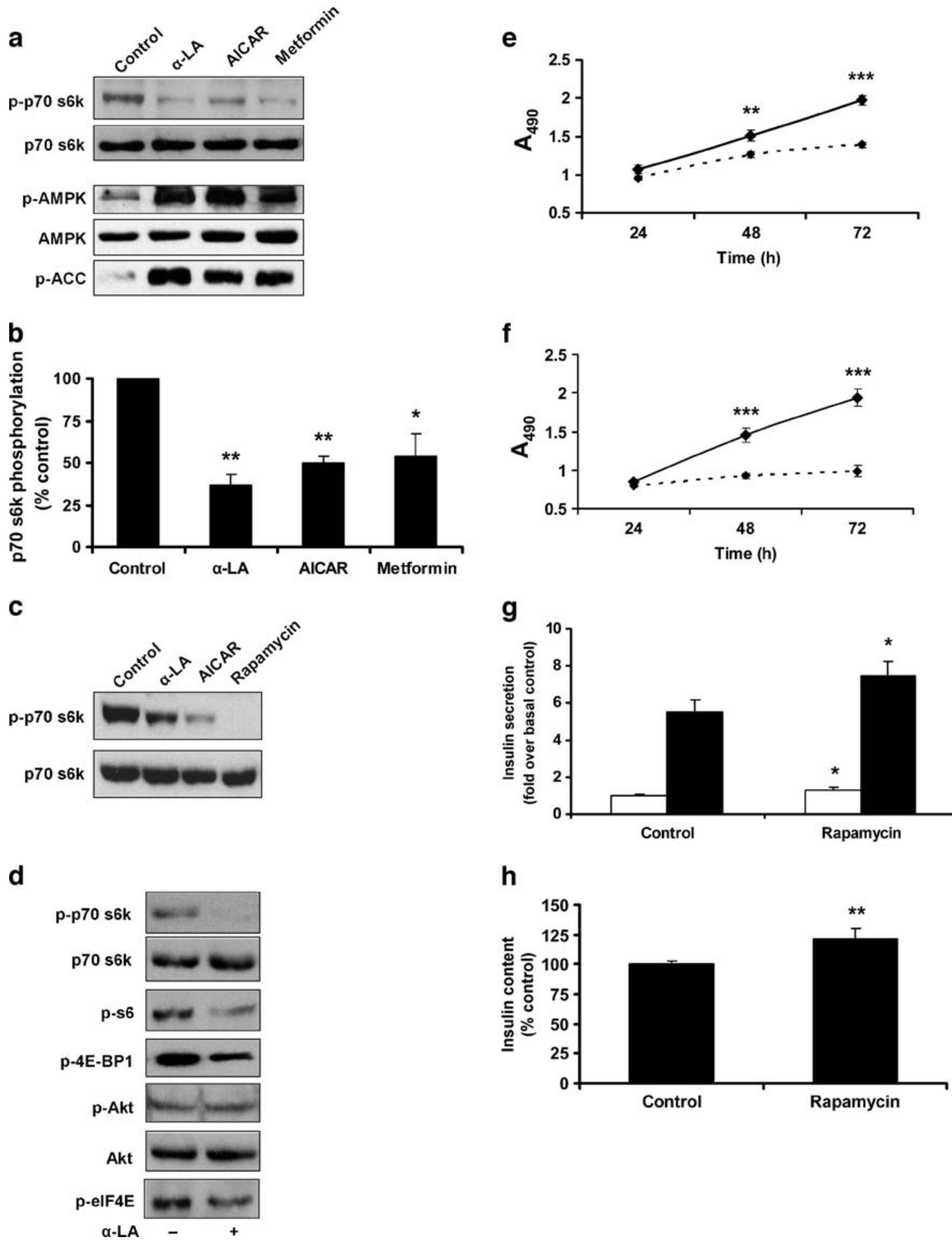

Fig. 6 α-LA negatively regulates mTOR/p70 s6k signalling. **a** MIN6 cells were serum-starved in 5 mmol/l glucose for 4–5 h and treated for 30 min with 10% serum and 25 mmol/l glucose in the presence or absence of 2.0 mmol/l α-LA, 2.0 mmol/l AICAR or 2.0 mmol/l metformin. Representative immunoblots are for phospho (p)- and total p70 s6k, as well as the AMPK/ACC pathway. **b** Relative changes in p70 s6k phosphorylation under the conditions in (**a**), represented as band density. Results are means±SE (n=3). **c** MIN6 cells were treated for 24 h with 2 mmol/l α-LA, 1 mmol/l AICAR or 5 nmol/l rapamycin. Blots are shown for phospho- and total p70 s6k. **d** MIN6 cells were treated for 30 min with 2.0 mmol/l α-LA in 25 mmol/l glucose and 10% serum. Representative blots are for the indicated proteins (n=3 independent experiments). **e**, **f** MIN6 cells were cultured in the presence (*dashed line*) or absence (*unbroken line*) of 2.0 mmol/l α-LA (**e**) or in the presence (*dashed line*) or absence (*unbroken line*) of 5 nmol/l rapamycin (**f**) for 24–72 h and cell growth was measured using an XTT assay (n=3). **g**, **h** Effect of rapamycin on insulin secretion from MIN6 cells. **g** Insulin secretion at 0 (*open bars*) or 15 mmol/l (*closed bars*) glucose from MIN6 cells treated for 24 h with 5 nmol/l rapamycin (n=3). **h** Total insulin content in MIN6 cells treated for 24 h with 5 nmol/l rapamycin (n=4). All secretion and total insulin data were normalised to total DNA and are expressed as means±SE. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with controls

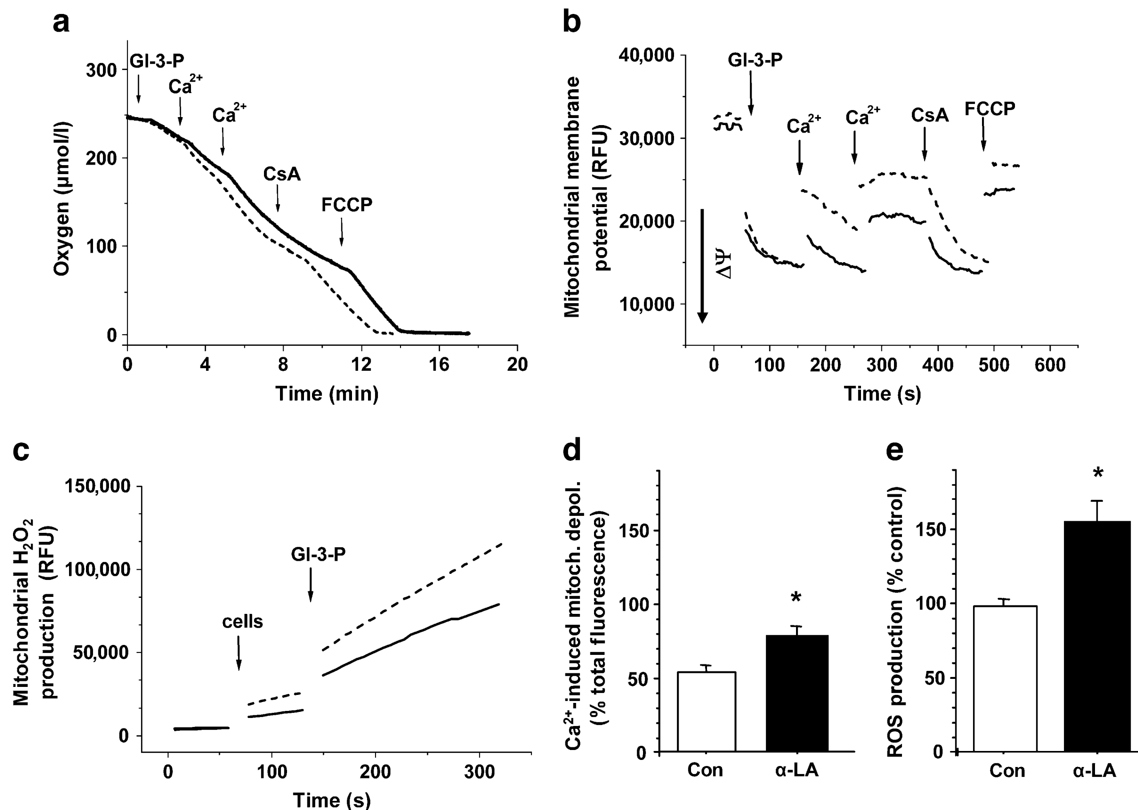

Fig. 7 Effect of α-LA on mitochondrial parameters in permeabilised MIN6 cells. MPT opening monitored by Ca^{2+}-induced oxygen consumption (**a**) and mitochondrial depolarisation (**b**) in the presence (*broken line*) or absence (*unbroken line*) of 200 µmol/l α-LA. For (**b**), increasing fluorescence corresponds to reduced $\Delta\Psi_m$. Representative traces are shown for each. **c** Sample traces of ROS measurements from cells treated with (*broken line*) or without (*unbroken line*) 200 µmol/l α-LA. *Arrows* (**a–c**) indicate the timepoints for addition of respiratory substrate glycerol-3-phosphate (*Gl-3-P*, 8 mmol/l), MPT inducer Ca^{2+} (50 µmol/l), MPT inhibitor cyclosporin A (*CsA*, 1 µmol/l) and mitochondrial uncoupler *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (*FCCP*, 1 µmol/l). *RFU* relative fluorescence units. All experiments were conducted at least three times with similar results. **d** Statistical data (from **b** and **c**) for mitochondrial depolarisation (*mitoch depol*) induced by 100 µmol/l Ca^{2+} expressed as a percent of total fluorescent signal, and (**e**) for rate of respiration-dependent ROS production expressed as a percent of control value. *p<0.05 compared with control (Con)

α-LA acted as an insulin sensitiser in skeletal muscle [38], and prevented endothelial cell dysfunction in obese rats [39], effects shown to be attributable to AMPK activation. Thus, it is likely that AMPK is responsible for many of the diverse antidiabetic effects of this compound.

The beneficial properties of α-LA, in particular as an antioxidant [1, 3, 4] and stimulator of glucose uptake, make this a potentially useful agent for diabetes therapy. However, we have shown here that α-LA inhibits insulin secretion in vitro from MIN6 cells and isolated rat islets. These findings are supported by other reports describing similar inhibitory effects with metformin or AICAR [21, 26, 27]. It is likely that the negative regulation of insulin secretion and positive effect on insulin sensitivity by these three drugs are mediated by AMPK. Several studies have shown that AMPK suppresses insulin secretion and synthesis in beta cells [19, 21, 22], consistent with its role as an energy-conserving enzyme. There are a number of possible mechanisms by which α-LA may inhibit insulin secretion through AMPK. This enzyme has been shown to have an important role in suppressing preproinsulin promoter activity [21]. It has also been demonstrated that activation of AMPK leads to modulation of secretory vesicle dynamics [22]. More recently, Richards et al. [40] showed that mouse islets infected with a constitutively active AMPK α-subunit had reduced glucose oxidation and insulin secretion, and were associated with poorer glycaemic control when transplanted into streptozotocin-diabetic mice compared with islets infected with null or dominant-negative AMPK viruses.

AMPK is an important regulator of the cellular response to nutrient availability, including protein translation and cell growth, and when activated, AMPK inhibits mTOR/p70 s6k signalling [31]. However, whether mTOR is involved in AMPK-mediated inhibition of insulin secretion is currently not known. Consistent with a positive role for p70 s6k in insulin secretion, mice lacking this enzyme have impaired GSIS and reduced beta cell mass [41]. We have shown in this report that α-LA inhibits mTOR/p70 s6k signalling and cell growth in MIN6 cells, and reduces GSIS and insulin content. While rapamycin and α-LA both caused inhibitory effects on cell growth and p70 s6k phosphorylation, the effect we observed with rapamycin on GSIS—a stimulatory one—is not consistent with a positive

role for mTOR in insulin secretion. In agreement with our data, one report demonstrated that rapamycin improved insulin secretion in vivo in dogs following islet autografts [42]. While others have shown that rapamycin has inhibitory effects in rat islets, these observations were made only at high doses and treatment times of greater than 3 days [43]. Another report demonstrated that rapamycin treatment (48 h) was detrimental to GSIS from HIT-T15 cells, but not rat islets treated for 24 h [44]. These discrepancies may be due to differential sensitivity to rapamycin in assorted cell lines or animal species, or variations in experimental protocols. The fact that p70 s6k knock-out mice have reduced GSIS [41] may be a reflection of a total-body loss of this enzyme and thus is likely to include contributions from the effects of p70 s6k deficiency in other tissues, including enhanced insulin sensitivity in the periphery [45]. More studies are required in order to clearly understand if rapamycin and/or mTOR exhibit any significant effect on insulin secretion, and if AMPK is involved in this process.

We demonstrated here that in addition to prolonged exposure to α-LA, acute treatment also inhibited insulin secretion from isolated rat islets, MIN6 cells and the perfused mouse pancreas. Previous reports [19, 21] suggested that acute AMPK activation inhibits GSIS. Although we cannot completely rule out this possibility, our data suggest that acute activation of AMPK was not exclusively responsible for our observations, given that AMPK and ACC phosphorylation were induced by AICAR, and to a lesser extent, metformin, yet these drugs did not have an acute inhibitory effect on insulin secretion (Fig. 5b,c). It is possible that potential non-specific effects of AICAR, or differences in cell lines or experimental protocols, could account for the differences observed here compared with those of the Hardie [19] and Rutter [21] laboratories. Our data indicate that α-LA may acutely inhibit insulin secretion via an alternative route involving direct actions on beta cell mitochondria—a process consisting of increased ROS production and induction of MPT opening. This would partially depolarise the mitochondria, thereby reducing the proton-motive force and thus ATP synthesis and, consequently, insulin secretion. Observations of increased ROS in isolated rat soleus muscle treated with α-LA [34], and ROS-induced MPT in rat liver mitochondria by α-LA [37], support our data that this compound can possess pro-oxidant properties. Our findings are important, given the clinical relevance of α-LA as an antioxidant [5]. It is possible that at particular concentrations, or in the absence of other pro-oxidants, α-LA itself acts as a pro-oxidant, whereas under conditions of oxidative stress, such as diabetes [5], this compound exhibits beneficial protective properties. As such, the clinical usefulness of α-LA may be contingent upon a particular pathophysiological state. It is not known if α-LA-induced ROS generation or mitochondrial depolarisation serves as a stimulus for AMPK activation by an upstream kinase such as LKB1 [12] or Ca^{2+}/calmodulin-dependent protein kinase kinase [46], but this remains an interesting possibility. Figure 8 depicts a potential scheme

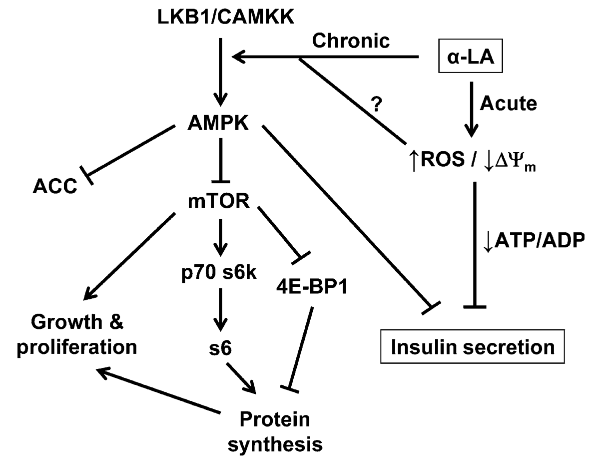

Fig. 8 Model of α-LA regulation of AMPK and beta cell function. Acutely, α-LA may inhibit GSIS by stimulating ROS and reducing $\Delta\Psi_m$, thus impairing the cellular response to glucose stimulation. These effects may also serve as a stimulus for AMPK activation by an upstream kinase such as LKB1 serine/threonine kinase (*LKB1*) or Ca^{2+}/calmodulin-dependent protein kinase kinase (*CAMKK*). AMPK activation subsequently inhibits insulin secretion and cell growth. *Arrows* represent activation or stimulation; *blunted lines* signify an inhibitory process. *4E-BP1* 4E-binding protein 1

by which α-LA exerts its multiple effects on beta cell function.

It can be argued that caution should be used when considering AMPK activators such as α-LA and metformin for the treatment of diabetes; there appears to be a trade-off of increased insulin sensitivity for reduced insulin secretion. However, simultaneous activation of AMPK in canonical insulin-sensitive tissues and the beta cell in a physiological state may be beneficial, as improved insulin use is accompanied by a smaller demand for insulin production, which may protect beta cells from exhaustion. Furthermore, while AMPK-activating compounds are thought to exert negative effects on GSIS under standard culture conditions, several reports suggest that AMPK can be protective in beta cells under pathological conditions. Marchetti et al. [47] demonstrated that metformin restores beta cell function and prevents apoptosis in diabetic human islets, while others reported beneficial properties for AICAR in clonal beta cells under lipotoxic conditions [48]. Whether α-LA behaves in a similar manner is an interesting possibility, especially if this compound would act as an antioxidant under these conditions.

In conclusion, our data reveal several novel pharmacological properties of α-LA, including the inhibition of insulin secretion from rat islets, the perfused mouse pancreas and MIN6 cells. α-LA also acts as a signalling molecule, activating AMPK and inhibiting p70 s6k and subsequently cell growth. α-LA directly modulates the mitochondrial state via an acute increase in ROS production and depolarisation of the mitochondrial membrane. Our findings provide some insight into a possible explanation for the insulin-sensitising properties of α-LA, which may occur in peripheral tissues in an AMPK-dependent manner. Further studies on α-LA and its relationship with AMPK are needed to gain a better

understanding of the antidiabetic properties of this molecule and the precise mechanism by which AMPK regulation occurs.

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