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ORIGINAL ARTICLE—ALIMENTARY TRACT

Metformin protects against lipoapoptosis and enhances GLP-1 secretion from GLP-1-producing cells

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Abstract
Background Metformin is the most frequently prescribed drug for treatment of type 2 diabetes. It improves insulin resistance and glycemia by reducing hepatic gluconeogenesis. In addition, diabetic patients on metformin therapy have elevated levels of the insulinotropic hormone glucagon-like peptide-1 (GLP-1) and metformin has been shown to regulate the expression of the GLP-1R in the pancreas.
Methods We have studied the direct long-term effects of metformin on apoptosis, and function of GLP-1-secreting L cells in vitro, using the murine GLUTag cell line as a model. The apoptosis of GLUTag cells was detected by DNA-fragment assay and caspase-3 activity determination. GLP-1 secretion was determined using ELISA and the expression of proglucagon mRNA was assessed by reverse transcription polymerase chain reaction. The activation of intracellular messengers was determined using western blotting.
Results Metformin significantly decreased lipotoxicity-induced apoptosis in conjunction with increased phosphorylated AMPK. Metformin also countered the JNK2 activation evoked by lipotoxicity. In addition, long-term metformin treatment stimulated GLP-1 secretion.
Conclusion This study demonstrates that metformin protects against lipoapoptosis (possibly by blocking JNK2 activation), and enhances GLP-1 secretion from GLP-1-producing cells in vitro. These direct effects of the drug might explain the elevated plasma GLP-1 levels seen in diabetic patients on chronic metformin therapy. The findings may also be harnessed to therapeutic advantage in efforts aiming at enhancing endogenous GLP-1 secretion in type 2 diabetic patients.

Keywords Glucagon-like peptide-1 · Gluconeogenesis · Insulinotropic · Lipotoxicity · L cell

Abbreviations

AMPK	AMP-activated protein kinase
JNK2	c-Jun N-terminal kinase
GLP-1	Glucagon-like peptide-1
PKA	Protein kinase A
PKC	Protein kinase C

Introduction

Type 2 diabetes (T2D) is characterized by hyperglycemia, resulting from impaired insulin production and insulin resistance in peripheral tissues [1]. The clinical management of T2D involves a combination of dietary treatment, application of oral antidiabetic drugs, and eventually also insulin replacement therapy in many cases. Under physiological conditions, glucose is the major stimulator of

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insulin secretion. Incretin hormones, such as glucagon-like peptide-1 (GLP-1), are produced by enteroendocrine L cells and augment meal-stimulated insulin secretion in a glucose-dependent manner [2]. More precisely, the incretin effect is the ability of gastrointestinal hormones such as GLP-1, released in response to food intake, to stimulate insulin release. In healthy individuals, this effect accounts for 50–70 % of prandial insulin secretion [3]. In T2D, the incretin response has been suggested to be impaired, as a result of reduced postprandial GLP-1 concentrations [4]. Administration of GLP-1 to T2D patients restores glucose-induced insulin secretion as well as the beta cells' sensitivity to glucose and normalizes fasting and postprandial glycemia [3].

Glucagon-like peptide-1 is synthesized in intestinal enteroendocrine L cells expressing the glucagon gene. Two major molecular forms of GLP-1 exist: GLP-1(7–36) amide and GLP-1(7–37). The majority of circulating biologically active GLP-1 is found in the GLP-1(7–36) amide form.

Native GLP-1 has a half-life of less than 2 min, owing to its degradation by dipeptidyl peptidase-4 (DPP-4) ubiquitously present in plasma, and this complicates its application as an antidiabetic drug. Stable analogues of GLP-1 and agents that inhibit the degradation of GLP-1(7–36) are available as treatments for T2D. However, enhancing endogenous incretin production/secretion by direct stimulation of GLP-1 secretion, along with long-term promotion of growth and differentiation and reduced apoptosis, of GLP-1-producing cells may be a novel option in incretin-based diabetes therapy (besides the current use of stable GLP-1 analogues and DPP-4 inhibitors), but this has—as of yet—not been explored to any appreciable extent.

Considering the significance of the incretin effect of GLP-1, long-term regulation of GLP-1-secreting cells becomes very important but has been relatively neglected in diabetes research.

Metformin is an oral antidiabetic cationic drug of the biguanide class and the most widely prescribed antidiabetic agent in the world. Metformin reduces blood glucose by direct effects on the liver and reduced gluconeogenesis as well as reduced insulin resistance. The mechanisms behind the antidiabetic actions of metformin are still unclear, although the best known target for metformin is the 5′ AMP-activated protein kinase (AMPK)—a fuel and stress-sensing enzyme that is considered a master switch of glucose and lipid metabolism in various organs [5].

Chronic metformin treatment of diabetic patients is associated with increased levels of circulating GLP-1 following an oral glucose load [6], but the mechanisms underlying this increase remain elusive. Considering the frequent use of metformin against T2D, the indicated effects on the incretin system and GLP-1, as well as the importance and proposed deficiency of GLP-1 in T2D, it becomes very important to understand how chronic metformin therapy modulates this part of the incretin–islet axis. Understanding this may also help to unravel some of the mechanisms regulating the enteroendocrine L cells, and may be a first step towards new and improved diabetes treatments based on increased endogenous GLP-1 secretion.

In the current study, we sought to determine whether metformin exerts direct long-term effects on the regulation of GLP-1-secreting cells in terms of apoptosis, and secretion, the nature of such effects, and the possible involvement of AMPK in some of these effects.

Materials and methods

Cell culture and in vitro exposure

The GLP-1-secreting GLUTag cell line (derived from a glucagon-producing enteroendocrine cell tumor that arose in transgenic mice generated on an out-bred CD-1 background) [7], graciously donated by Dr. Neil Portwood of the Karolinska Institutet, Solna, Sweden, and originally from Dr. Daniel J. Drucker, Mount Sinai Hospital, Samuel Lunenfeld Research Institute, University of Toronto, Canada, was cultured in DMEM (Invitrogen Inc., Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 5.5 mM glucose, 10,000 U/ml penicillin, and 10 mg/ml streptomycin sulfate (Invitrogen Inc.) under 5 % CO_2. Palmitate (sodium palmitate, Sigma-Aldrich) exposure medium was supplemented with 0.5 % bovine serum albumin (BSA, fatty acid free) (Sigma-Aldrich). Palmitate was dissolved in 12.5 % ethanol during heating to 60 °C. Control cells were given vehicle with equal amounts of ethanol as the palmitate-exposed cells (final concentration of ethanol 0.03 %). Metformin, 5-aminoimidazole-4-carboxamide ribotide (AICAR), and compound C were purchased from Sigma-Aldrich.

MTT assay

GLUTag cells were plated and cultured in 96-well plates at a density of 136,000 cells/ml for 24 h. Cells were then washed and treated with or without 0.125 mM palmitate for 48 h in the presence of low serum medium (2 % FBS). Viable cell densities were determined by metabolic conversion of the dye MTT. Thus, 15 µl of the supplied MTT solution was added to each well and the plates were then incubated for an additional 4 h. The MTT reaction was terminated by the addition of 100 µl acidified isopropanol,

dissolving the formazan product formed. After 1–2 h at 4 °C, MTT assay results were read by measuring the absorption at 540 nm. Each experiment was performed in six duplicates and repeated at least three times to assess the consistency of results.

Protein assay

GLUTag cells were washed twice with phosphate buffered saline (PBS) and lysed on ice in a RIPA lysis buffer containing 150 mM NaCl, 20 mM Tris, 0.1 % SDS, 1 % Triton X-100, 0.25 % Na-deoxycholate, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, and protease inhibitory cocktail (Sigma-Aldrich) for 30 min. Samples were clarified by centrifugation, supernatants were transferred to new tubes, and the total protein concentration was determined with the Bio-Rad DC protein assay (method of Lowry et al. [8], using BSA as a standard) (Bio-Rad Laboratories, Hercules, CA).

Apoptosis assays

DNA fragmentation ELISA

GLUTag cells were plated (at a density of 180,000 cells/ml) and grown in 24-well plates for 24 h. Cells were then washed twice with low serum medium (2 % FBS, 5.5 mM glucose) and then treated with metformin at indicated doses in 2 % FBS and 5.5 mM glucose medium for an additional 48 h. DNA fragmentation ELISA (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer's instructions. Briefly, the cells were lysed and lysates and reagents were added as described by the manufacturer and the ELISA plate was read by measuring absorption at 450 nm. All experiments were performed in triplicates and repeated twice to assess the consistency of response.

Caspase-3 activity assay

GLUTag cells were plated (at a density of 250,000 cells/ml) and grown in Ø60-mm Petri dishes for 24 h. Cells were then washed twice with low serum medium (2 % FBS, 5.5 mM glucose) and then treated with metformin at indicated doses in 2 % FBS and 5.5 mM glucose for an additional 48 h. Caspase-3 activity assay kit (Cell Signaling Technology, Inc., Danvers, MA) was performed according to the manufacturer's instructions. Briefly, the caspase-3 colorimetric assay is based on the hydrolysis of a substrate by caspase-3, resulting in the release of fluorescent product, which can be measured at 405 nm.

Hormone secretion/content assays

GLUTag cells were plated (at a density of 180,000 cells/ml) and grown in 24-well plates for 24–48 h. Cells were then treated with metformin at indicated doses for an additional 48 h. Immediately after the 48-h incubation, medium was collected and DPP-4 inhibitor added (10 μl/ml) (Millipore Corporation, Billerica, MA). The remaining medium was discarded and the cells were washed with prewarmed KRBH buffer/0.2 % BSA/0 mM glucose, followed by a 30-min pre-incubation with the same buffer. Cells were then treated with 20 mM glucose, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (PKC activator) (Sigma-Aldrich), and forskolin (PKA activator) (Sigma-Aldrich) for 2 h. Immediately thereafter, DPP-4 inhibitor (Millipore Corporation) was added and the buffer was collected. Cells were lysed using RIPA lysis buffer (Bio-Rad Laboratories) and lysates analyzed for GLP-1 content normalized to total protein content (to control for equal numbers of cells). GLP-1 Active ELISA (Millipore Corporation) was performed according to the manufacturer's instructions. All experiments were performed in triplicates and repeated at least three times to assess the consistency of results.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

GLUTag cells were lysed and RNA extracted using Aurum total RNA mini kit (cat # 7326820) (BioRad Laboratories) according to the manufacturer's instructions. cDNA was synthesized for qPCR using iScript™ cDNA synthesis kit (BioRad Laboratories) according to the manufacturer's instructions.

Proglucagon primers were designed according to GATTTTGTGCAGTGGTTGAT, ACTTCTTCTGGGAA GTCTTCG, using Invitrogen custom primer design software (Invitrogen, Inc).

A one-step RT-PCR kit with SYBR Green (iScript™ one-step RT-PCR kit with SYBR® Green) (BioRad Laboratories) was used for real-time quantitative RT-PCR. This kit utilizes iScript RNase H+ reverse transcriptase and hot-start iTaq DNA polymerase. GAPDH was used as housekeeping gene for normalization.

Western blot analysis

GLUTag cellular protein was extracted following culture with or without metformin and AICAR in the presence or absence of palmitate–albumin complex at 2 % serum DMEM, using RIPA lysis buffer (Bio-Rad Laboratories) for 30 min on ice. Cells were sonicated and lysates were cleared by centrifugation. Protein concentration was determined and cell extracts were stored at −80 °C. Equal amounts of protein were then mixed with reducing SDS-PAGE sample buffer, boiled for 5 min, and proteins were separated by SDS-PAGE. Samples containing 25–30 μg of protein were electrophoresed against a pre-stained protein

ladder (Sigma Aldrich, SM 1811) on a 10 % polyacrylamide gel under denaturing conditions, followed by transfer to PVDF membrane (Bio-Rad Laboratories). Membranes were blocked with 5 % milk solids in PBST; primary (ON) and secondary (1 h) antibody incubations were performed in the same buffer, with three 10-min washes in PBS-T intervening. Anti-phospho-AMPK and anti-AMPK antibodies were from Abcam, Cambridge, UK.

Horseradish peroxidase-conjugated secondary antibodies (1:5,000) (Santa Cruz Biotechnology, CA) and ECL (enhanced chemiluminescence) (GE Healthcare, Fairfield, CT) reagents were used to detect proteins. Images and quantifications were obtained using Molecular Imager ChemiDoc XRS with Quantity One Software v. 4.6.5 (Bio-Rad Laboratories). After imaging, the PVDF membranes were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories) for total protein normalization. Phosphorylation was determined after normalization with total (phosphorylated and non-phosphorylated) forms of the protein or Coomassie staining.

Statistical analysis

Comparisons between groups, treatments, and time were made by a one-way ANOVA for repeated measures. Significant differences by ANOVA were followed by post hoc Sheffé test. Comparisons between control and single treatment groups were done using two-tailed Student's *t* test. $P < 0.05$ was deemed statistically significant. Power analysis was performed and taken into consideration for all experiments performed.

Results

Metformin protects GLP-1-producing cells against lipoapoptosis and blocks lipotoxicity-induced caspase-3 activity

Acute stimulation with the FFA palmitate dose-dependently stimulated GLP-1 secretion (Fig. 1a). However, we also aimed to investigate how persistently elevated levels of FFAs influence the GLP-1-secreting cells in terms of apoptosis. To mimic the effect of diabetic hyperlipidemia on cell viability, GLUTag cells were treated in the presence of the fatty acid palmitate (0.125 mM) for 48 h in 2 % FBS. The results show that the palmitate treatment decreased GLUTag cell viability by 30 % (Fig. 1b) and significantly increased DNA fragmentation (Fig. 1c), indicative of lipoapoptosis. This serum concentration was chosen as it did not induce "starvation", as evident by morphology and growth of the cells, but prevented a very high serum concentration from "masking" possible effects of a pharmacological agent such as metformin. Co-incubation with metformin (500 μM, 1 mM, 2 mM), preceded by a 45-min pre-incubation with the drug, dose-dependently blocked palmitate-induced DNA fragmentation (Fig. 1c), with a significant effect of co-incubation with 2 mM metformin.

Palmitate significantly increased the catalytic activity of caspase-3 in the GLUTag cells, with a twofold increase detected after 48 h (Fig. 1d). Co-incubation with 2 mM metformin completely abolished lipotoxicity-induced activation of caspase-3, indicating protection against lipoapoptosis, whereas metformin alone did not affect caspase-3 activity (Fig. 1d). However, co-incubation with 500 μM metformin did not influence palmitate-induced caspase-3 activity (Fig. 1d).

Metformin inhibits palmitate-induced JNK phosphorylation with a simultaneous phosphorylation of AMPK

We hypothesized that the lipoapoptosis noted may be mediated by effects on the mitochondria and an intrinsic pathway with activation of downstream components, such as c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK). Thus, we determined the phosphorylation of JNK and ERK after treatment with palmitate using western blotting at different time points (20 min, 1 h, 8 h). ERK phosphorylation was not affected by palmitate treatment (109 ± 20, 114 ± 5, 104 ± 11 % of basal phosphorylation at 20 min, 1 h, 8 h, respectively). However, after 8 h there was an increase in the phosphorylation of JNK in response to 0.125 mM palmitate that was significantly attenuated by co-incubation with 2 mM metformin (Fig. 2a, b). To further determine the role of JNK in lipoapoptosis, we pre-treated the cells with the JNK inhibitor SP600125 and determined palmitate-induced caspase-3 activity. In accordance with a lipoapoptosis mediated at least in part by JNK activation, the JNK inhibitor significantly reduced palmitate-induced caspase-3 activity (Fig. 2c).

Further, we determined that the reduction in JNK phosphorylation in response to metformin occurred with a simultaneous metformin-induced phosphorylation (activation) of AMPK (Fig. 2d, e).

AICAR-induced AMPK activation does not replicate the prevention of lipoapoptosis by metformin

To investigate if the lipoprotective effect seen in the GLUTag cells in response to metformin is mediated by its activation of AMPK, we used the specific AMPK activator AICAR to evaluate if we could reproduce the protective

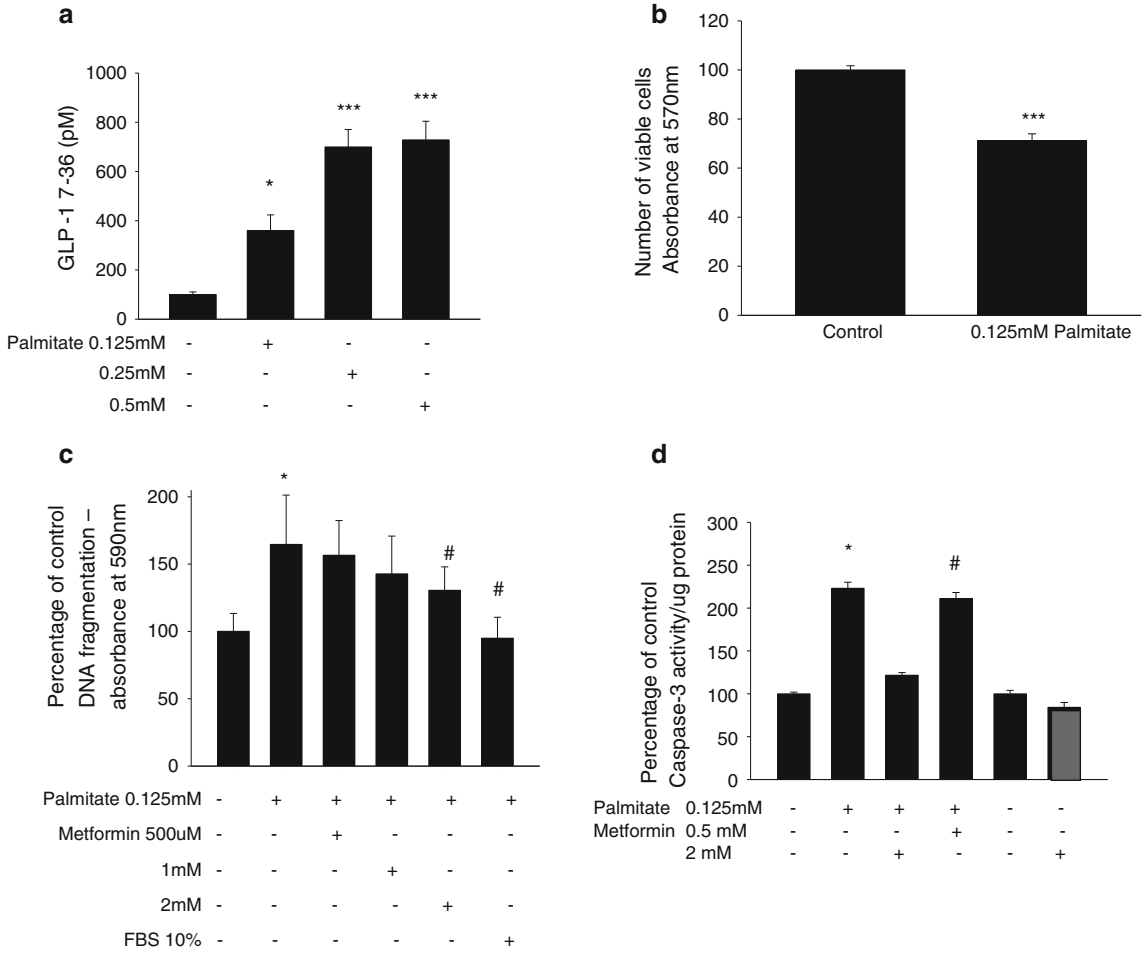

Fig. 1 Metformin stimulates GLP-1 secretion and protects GLP-1-producing cells against lipoapoptosis. **a** A 2-h treatment with palmitate dose-dependently increases GLP-1 secretion. **b** Palmitate (0.125 mM) decreased GLUTag cell viability after a 48-h incubation, as measured by MTT assay ($n = 3$, in duplicates). **c** Palmitate (0.125 mM) significantly increased GLUTag cell DNA fragmentation after a 48-h incubation, whereas co-incubation with increasing concentrations of metformin reduced the palmitate-induced DNA fragmentation, where a statistically significant effect was seen in response to 2 mM metformin, as measured by DNA fragmentation ELISA. The protective effect of 10 % fetal bovine serum was used as a positive control ($n = 3$; in triplicates). **d** Palmitate increased GLUTag cell caspase-3 activity after a 48-h incubation, whereas this effect was counteracted by co-incubation with 2 mM metformin. Co-incubation with 500 μM metformin could not reduce the palmitate-induced caspase-3 activity after a 48-h incubation ($n = 3$–7, in duplicates). *Bars* represent mean ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ compared with controls. $^{\#}p < 0.05$ compared with palmitate-treated cells

effect of metformin. However, pre-incubation with AICAR failed to reproduce the lipoprotective effect of metformin (Fig. 3a). When assessing the phosphorylation of AMPK induced by AICAR (200 μM) and metformin (500 μM, 2 mM), we found that both agents produced a rapid and transient AMPK activation, detectable already after 20 min and still detectable after 1.5 h. However, metformin-induced AMPK phosphorylation reached its peak later than AICAR-induced phosphorylation—after 1.5 h as compared to 20 min (Fig. 3b, c). Western blotting failed to show phosphorylation of AMPK in response to the agents after 4 h (data not shown). However, 2 mM metformin induced a second phase of AMPK phosphorylation at 8 (Fig. 2d) and 24 h (Fig. 3d). In contrast, no AMPK activation could be detected in response to 200 μM AICAR after 24 h (Fig. 3d,e).

The partial block of AMPK phosphorylation by compound C attenuates the lipoprotective effect of metformin

To determine the role of this second phase/sustained AMPK phosphorylation observed in response to 2 mM metformin (Fig. 3c) in the lipoprotective effect of the drug, we used a pre-incubation with 2 mM metformin in the presence or absence of the specific AMPK inhibitor compound C and determined the effect on apoptosis induced by palmitate by measuring the activity of cleaved caspase-3.

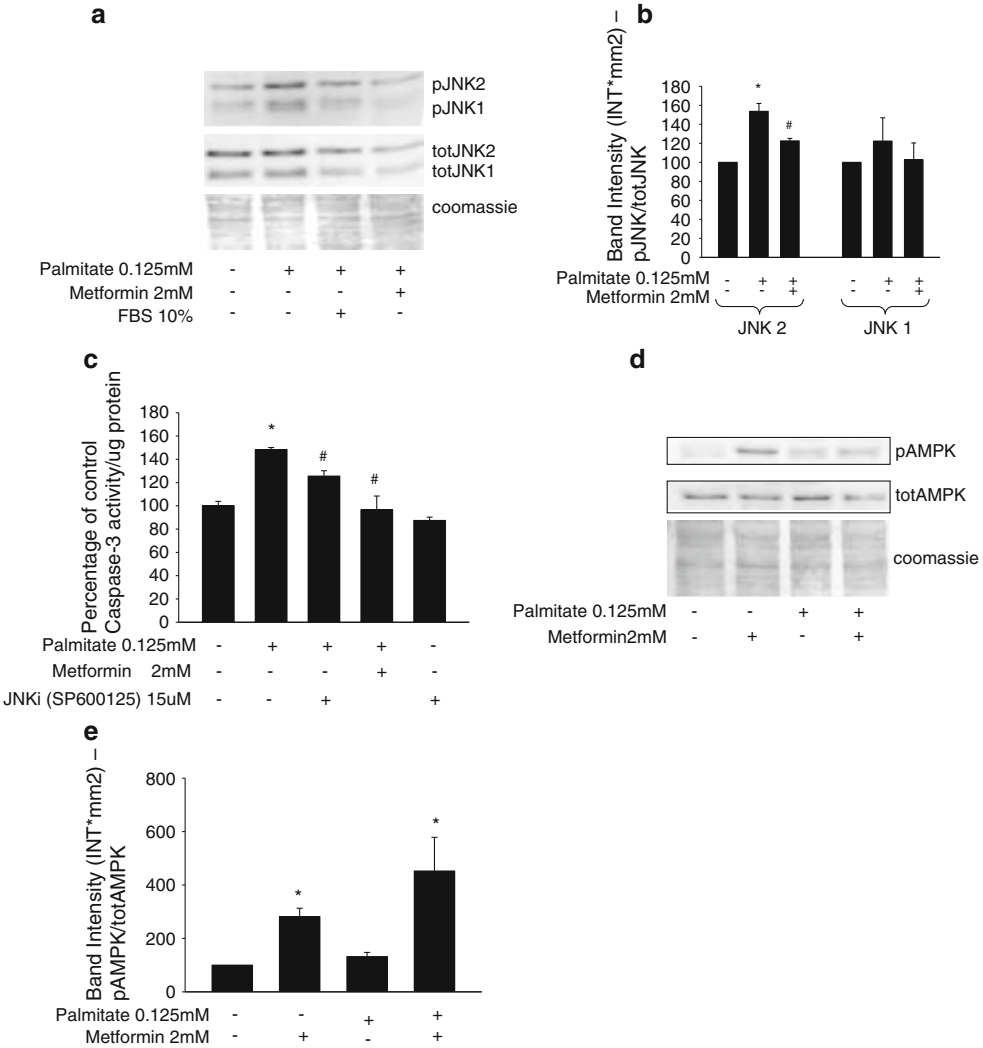

Fig. 2 Metformin inhibits lipotoxicity-induced JNK phosphorylation and activates AMPK in GLP-1-secreting cells. Representative blot (**a**) and statistical analysis (**b**) showing that 8-h co-incubation with 2 mM metformin attenuates palmitate-induced phosphorylation of JNK ($n = 3$). Co-incubation with the JNK inhibitor SP600125 reduces caspase-3 activity in response to palmitate (**c**). Representative blot (**d**) and statistical analysis (**e**) showing that 8-h incubation with 2 mM metformin phosphorylates AMPK ($n = 3$). *Bars* represent mean ± SEM. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared with palmitate-treated cells

Co-incubation with the AMPK inhibitor resulted in an attenuation of the lipoprotective effect of metformin by approximately 50 %, as measured by comparing the difference in caspase-3 activity between palmitate alone and palmitate with metformin when agents are added to incubation medium containing compound C, as opposed to the difference in caspase-3 activity between the two treatments when performed in the absence of compound C (Fig. 4a). To determine if these data represented only a partial inhibition of AMPK phosphorylation induced by compound C, or an indication that the lipoprotective effect of metformin is only partially AMPK-dependent, we evaluated the inhibition of AMPK activity induced by compound C using western blotting analysis of the phosphorylation of the downstream AMPK substrate acetyl-CoA carboxylase (ACC). These results showed partial (45 %) inhibition of AMPK activation by compound C (Fig. 4b, c), in line with a 50 % decrease in the lipoprotective effect mediated by metformin, should such an effect be AMPK-dependent.

Metformin increases GLP-1 secretion but not expression of pre-proglucagon

To study GLP-1 secretion, we used GLUTag cells cultured under standard culturing conditions (in the presence of 10 % FBS). To validate GLUTag cells as a suitable model of GLP-1-producing cells in terms of GLP-1 secretion, the cells were treated with activators of protein kinase A (forskolin) and protein kinase C (TPA), known to stimulate acute GLP-1 secretion from native GLP-1-secreting cells. The GLP-1 secretory response induced by both TPA and forskolin (data not shown) was in agreement with that previously reported [9, 10]. In addition, glucose induced a secretory response, peaking at a very low concentration (1 mM) of glucose (data not shown), in line with what is reported for GLUTag cells [11]. The next step was to investigate whether metformin treatment—acute or long term—could alter GLP-1 secretion. We started out by investigating the effect of acute (30 min) incubation with

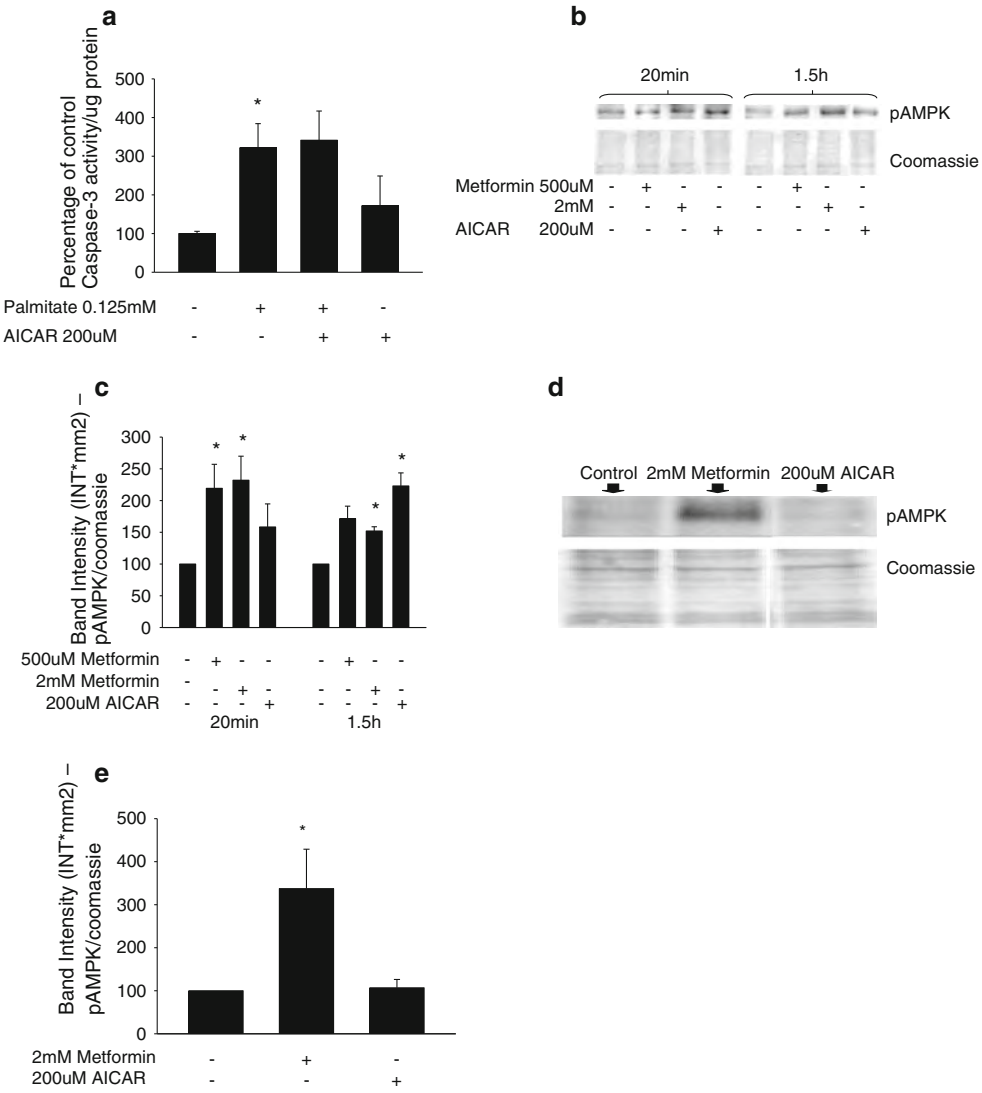

Fig. 3 AICAR-induced AMPK activation does not replicate the protective effects of metformin against lipoapoptosis in GLP-1-secreting cells. **a** Palmitate-induced GLUTag cell caspase-3 activity after a 48-h incubation is not affected by co-incubation with 200 μM AICAR ($n = 4$, in duplicates). Representative blot (**b**) and statistical analysis (**c**) showing the stimulatory effects of two concentrations of metformin and AICAR on the phosphorylation of AMPK as illustrated by western blotting after a 20-min (*left*) and 1.5-h (*right*) incubation ($n = 3$). Representative blot (**d**) and statistical analysis (**e**) showing the effect of 2 mM metformin and AICAR on the phosphorylation of AMPK as illustrated by western blotting after 24 h ($n = 3$). *Bars* represent mean ± SEM. $^*p < 0.05$

500 μM metformin on GLP-1 secretion from the GLUTag cells. However, this did not alter GLP-1 secretion (87 ± 5 % of basal secretion). In contrast, long-term treatment (48 h) with 500 μM metformin led to a significant increase in the amount of GLP-1 secreted into the medium (Fig. 5a, b). However, pre-proglucagon expression was reduced in response to the same concentration of the antidiabetic drug (Fig. 5c).

Discussion

In this study, we show for the first time that the antidiabetic drug metformin has direct long-term effects on the regulation of enteroendocrine GLP-1-secreting cells in vitro. Although we could confirm a direct dose-dependent stimulatory effect of acute stimulation with an FFA on GLP-1 secretion, in line with what has been previously observed [12], to our knowledge there is no information on how chronically elevated levels of FFAs influence the enteroendocrine GLP-1-secreting cells in terms of apoptosis. T2D patients often have elevated levels of plasma FFAs [13, 14], and high levels of FFAs induce insulin resistance and are toxic to many cell types. Specifically, FFAs acutely stimulate insulin secretion from pancreatic beta cells, whereas impaired beta cell function and lipotoxicity following long-term exposure have been reported [15].

The fatty acid palmitate is often used to simulate hyperlipidemia in vitro and has been reported to mediate apoptosis through an intrinsic pathway characterized by permeabilization of mitochondria and release of cytochrome *c* [16].

As plasma GLP-1 levels have been reported to be decreased in T2D patients, we wanted to evaluate whether high concentrations of fatty acids would induce apoptosis of the GLP-1-secreting cells. Palmitate was used to evaluate the effect of high concentrations of FFAs relevant to T2D, as it is the most abundant saturated FFA bound to

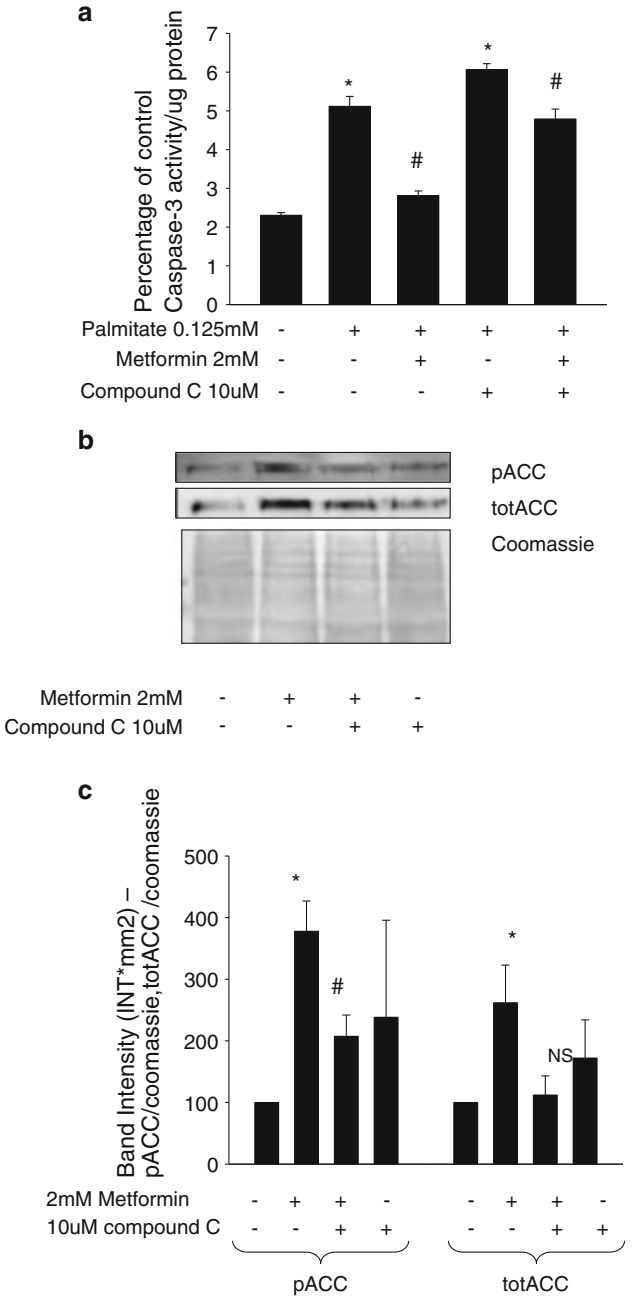

Fig. 4 Partially blocking the sustained AMPK phosphorylation using compound C also partially blocks the lipoprotective effect of metformin in GLP-1-secreting cells. Co-incubation with 10 μM compound C partially blocked the metformin-induced activation of AMPK, measured as phosphorylation of its downstream substrate ACC, as illustrated by western blotting. **a** Representative blot. **b** pACC and totACC band intensity normalized by Coomassie ($n = 4$). *Bars* represent mean ± SEM. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared with metformin-treated cells. **c** Co-incubation with 10 μM compound C partially blocks the lipoprotective effect of 2 mM metformin as measured by caspase-3 activity ($n = 3$, in duplicates). *Bars* represent mean ± SEM. $^{*}p < 0.05$ compared with control. $^{\#}p < 0.05$ compared with palmitate-treated cells. *NS* no significant difference compared to control

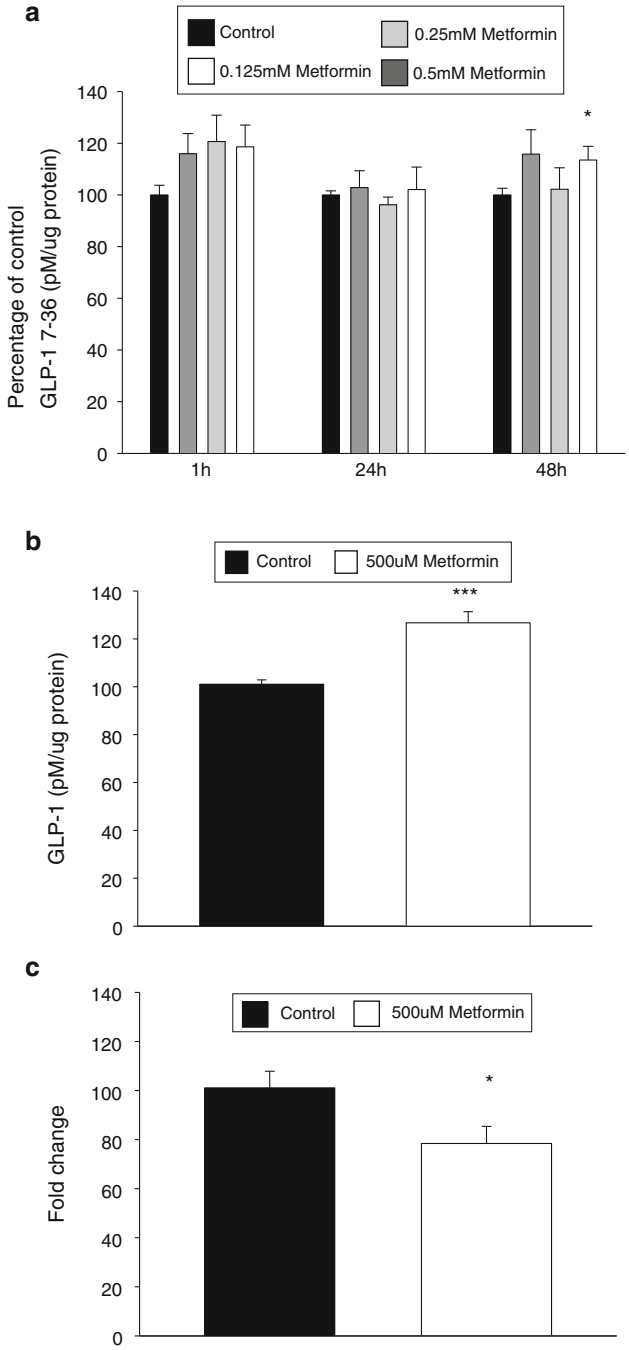

Fig. 5 Metformin increases GLP-1 secretion but not expression of pre-proglucagon. **a**, **b** While there was an indicated dose-dependent stimulatory effect of metformin on secretion in the presence of nutrient-supplemented medium, this effect was significant after a 48-h incubation with 500 μM metformin. **c** A 48-h incubation with 500 μM metformin reduced the expression of proglucagon mRNA ($n = 3$, in duplicates). *Bars* represent mean ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$ compared with controls

human serum albumin [17]. We used complexes where the molar ratio of FFA to albumin was 2:1, which is physiologically relevant. In addition, normal circulating FFA levels are approximately 0.5 mM [18], so the

concentrations of individual FFA species (0.125 mM) used are reasonable and relevant to the clinical situation. Our results demonstrate that palmitate induces massive cell death in the GLP-1-secreting cells. Further, we report that metformin protects GLP-1-secreting cells from this lipotoxicity. Whether this lipoapoptosis contributes to the GLP-1 deficiency in T2D patients, in whom lipotoxicity often prevails, and whether such an effect could be counteracted by metformin, will need to be further studied.

As to the molecular mechanisms underlying this lipotoxicity, we first studied JNKs, which are downstream components of the mitochondrial death signal [19]. JNK has also been reported to mediate palmitate-induced apoptosis in other cell systems [20]. We show here that palmitate treatment induces phosphorylation of JNK2 in the GLP-1-secreting cells, an effect that is significantly attenuated by co-treatment with metformin. Further, the JNK inhibitor SP600125 significantly attenuated palmitate-induced caspase-3 activity. However, the fact that SP600125 could not, like metformin, completely block palmitate induced caspase-3 activity indicates the involvement of additional signaling pathways. In the future we plan to also assess the involvement of another MAPKK, p38, and the effect on antiapoptotic markers such as Bcl-2 and the Akt signaling pathway.

The metformin-induced attenuation of JNK activation occurred in conjunction with a significant AMPK activation by metformin; knowing that AMPK activation is a well-known metformin action, we addressed whether AMPK was necessary for the lipoprotective effect of metformin. Surprisingly, the AMPK activator AICAR and a lower concentration of metformin (500 μM) failed to reproduce the lipoprotective effect of 2 mM metformin in the GLP-1-secreting cells. However, our data show differential activation of AMPK in response to 2 mM metformin and AICAR, in that both agents will induce rapid and transient phosphorylation of AMPK, but only 2 mM metformin will induce a later sustained activation of AMPK. The two phases of AMPK phosphorylation reported here are in agreement with what has been reported in other studies [21]. Thus, we hypothesize that, if the lipoprotective effect of metformin is AMPK-dependent, it requires a sustained activation of AMPK. In support of this hypothesis, the need for sustained AMPK activation in counteracting palmitate-induced apoptosis has been reported in other cell systems [22]. We also found that co-incubation with the AMPK inhibitor compound C resulted in an attenuation of the lipoprotective effect of metformin by approximately 50 %, which agrees well with the partial (50 %) inhibition of sustained AMPK activation achieved by the co-incubation with compound C, and an AMPK-dependent effect.

In this study, we also the determined direct effects of metformin on GLP-1 secretion. We found that long-term treatment with 500 μM metformin in the presence of nutrient-supplemented medium significantly stimulates GLP-1 secretion. However, the same concentration of metformin significantly reduces the transcription of pre-proglucagon and proglucagon mRNA expression. These data indicate that metformin has differential but direct effects at the transcriptional level and the level of translation/secretion. Interestingly, PKC is a target of metformin action [23], and activators of PKC have been shown to stimulate secretion, but not biosynthesis, of the proglucagon-derived peptides in GLUTag cell cultures [10]. It may also be that treatment with metformin sensitizes the GLUTag cells to GLP-1 secretagogues present in the cell culture medium, such as glucose. These are nevertheless pure speculations, and further studies are needed to determine the underlying mechanisms. However, a stimulatory secretory effect/enhanced nutrient-stimulated secretion by metformin would be of potential therapeutic importance because the reduced GLP-1 levels seen in T2D patients [4] have been reported to result from defective secretion of the hormone and not from a transcriptional defect [24, 25].

The enhanced GLP-1 secretion and protection against lipoapoptosis reported here by long-term treatment with metformin at low concentrations may provide an explanation for the increased plasma GLP-1 levels seen in diabetic patients on chronic metformin treatment [6]. Further, considering the impaired GLP-1 secretion in T2D, contributing to defective postprandial insulin secretion, it would be highly desirable to increase endogenous GLP-1 secretion. Protecting these cells from apoptosis induced by high levels of FFAs could contribute to an increased L cell mass and increased GLP-1 secretion. Such studies in vivo are under way in our laboratory.

Throughout this study we used the GLP-1-secreting GLUTag cells as a model. GLUTag cells are a stable immortalized murine enteroendocrine cell line that expresses the proglucagon gene and secretes the glucagon-like peptides [7]. In contrast to mouse STC-1 cells, GLUTag cells appear quite well differentiated, and recapitulate the responsiveness of primary intestinal cell cultures to physiological and pharmacological GLP-1 secretagogues [9, 10]. The GLUTag cells is thus one of the very few and best models available for studying the L cells. Since native L cells are very scarce and dispersed along the gastrointestinal tract as single cells it would be extremely difficult to study their growth and apoptosis in vitro. In future work we plan to address this important issue in vivo by feeding rodents metformin and a high fat diet, followed by immunohistological evaluation of L cell mass, growth, and apoptosis.

The concentrations of metformin (0.5–2 mM) used in these in vitro studies are supratherapeutic. The maximum recommended clinical metformin dose is 3,000 mg/day and

20 μM has been calculated as a clinically equivalent serum concentration in vitro [26, 27]. However, it is important to consider that cells in culture are grown in an environment of overabundant nutrients; 2–10 % FBS is added to the cell culture media, resulting in excessive growth stimulation. This is an inherent limitation of in vitro studies using cell lines and may explain why higher concentrations are needed to see the effects of metformin in cell culture than what is typically seen in diabetic patients. However, we must also take into consideration that metformin has been shown to accumulate in tissues at higher concentrations than in blood [28]. Additionally, as the L cells directly face the intestinal lumen, they may locally be exposed to very high metformin concentrations.

In summary, despite the differences in energy supply and dosing between these in vitro studies and clinical metformin use, metformin's lipoprotective potential in GLP-1-producing cells reported herein warrants thorough assessment for T2D treatment.

More data are needed to confirm the molecular mechanisms mediating the direct effects of metformin on GLP-1-secreting cells. Although we present data in support for an AMPK-dependent lipoprotective effect of metformin, we realize the limitations of using a relatively unspecific inhibitor of AMPK such as compound C, and emphasize the importance of confirming these data by using siRNA targeting AMPK and subsequent assessment of the effects, which is another of our future research goals.

In conclusion, although more data are needed to determine the physiological relevance and molecular mechanisms of the direct effects of metformin on the enteroendocrine GLP-1-secreting cells, this study provides novel and intriguing results suggesting that metformin by direct long-term effects on GLP-1-secreting cells promotes secretion and confers protection from diabetic lipoapoptosis of these cells.

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