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Molecular mechanisms of lipoapoptosis and metformin protection in GLP-1 secreting cells

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ABSTRACT

Background: Evidence is emerging that elevated serum free fatty acids (hyperlipidemia) contribute to the pathogenesis of type-2-diabetes, and lipotoxicity is observed in many cell types. We recently published data indicating lipotoxic effects of simulated hyperlipidemia also in GLP-1-secreting cells, where the anti-diabetic drug metformin conferred protection from lipoapoptosis. The aim of the present study was to identify mechanisms involved in mediating lipotoxicity and metformin lipoprotection in GLP-1 secreting cells. These signaling events triggered by simulated hyperlipidemia may underlie reduced GLP-1 secretion in diabetic subjects, and metformin lipoprotection by metformin could explain elevated plasma GLP-1 levels in diabetic patients on chronic metformin therapy. The present study may thus identify potential molecular targets for increasing endogenous GLP-1 secretion through enhanced viability of GLP-1 secreting cells in diabetic hyperlipidemia and obesity.

Methods: We have studied molecular mechanisms mediating lipotoxicity and metformin-induced lipoprotection in GLP-1-secreting L-cells *in vitro,* using the murine GLUTag cell line as a model. Diabetic hyperlipidemia was simulated in this cell system by addition of the fatty acid palmitate. Caspase-3 activity was used as a measure of GLUTag cell apoptosis. ROS production was determined using a fluorescent probe, and the activation of intracellular signaling pathways was assessed by Western blotting.

Results: Palmitate increased ROS production in GLP-1 secreting cells, and the lipotoxic effects of palmitate were abolished in the presence of the antioxidant Trolox. Further, palmitate phosphorylated p38 and inhibition of p38 using the p38 inhibitor SB203580 significantly reduced palmitate-induced caspase-3 activity. Pre-incubation of palmitate with metformin further increased palmitate induced ROS production, while significantly reducing the expression of p38.

Conclusion: This study demonstrates that palmitate induces ROS production and that the palmitate induced lipotoxicity is the result of increased ROS production, where the ROS sensitive MKK3/6-p38 pathway mediates lipoapoptosis of GLP-1-secreting cells. Further, in the presence of simulated hyperlipidemia, metformin increases ROS production. However, metformin significantly decreases the expression of p38, indicating that metformin mediated lipoprotection involves reduced activity of the p38 signaling pathway.

1. Introduction

Obesity increases the risk of developing type 2 diabetes (T2D); the increase of saturated fats in our diet, high caloric intake and lack of exercise has made T2D spread like an epidemic in today's society. T2D is characterized by hyperglycemia, resulting from impaired insulin production and insulin resistance in peripheral tissues [1]. Clinical management of T2D involves a combination of dietary treatment, application of anti-diabetic drugs and eventually also insulin replacement therapy in many cases. Under physiological conditions, glucose is the major stimulator of 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]. The incretin effect is defined as the ability of gastrointestinal hormones, such as GLP-1, released in response to food intake, to stimulate insulin release. In healthy individuals, it accounts for 50–70% of prandial insulin secretion [3]. In T2D, where hyperlipidemia and obesity often prevails, the incretin response has been

Abbreviations: AMPK, AMP-activated protein kinase; JNK2, c-Jun N-terminal kinase; GLP-1, glucagon-like peptide-1; ROS, reactive oxygen species; MAPKK, mitogen-activated protein kinase kinase.

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suggested to be impaired, as a result of reduced postprandial GLP-1 concentrations [4]. In addition, the GLP-1 secretory response is progressively diminished with increasing BMI [5]. However, administration of GLP-1 to T2D patients can normalize fasting and postprandial glycemia [3], but its application as an antidiabetic drug is made difficult by rapid degradation. Native GLP-1 has a half-life of less than 2 min, due to degradation by dipeptidyl peptidase-4 (DPP-4) ubiquitously present in plasma. Stable analogs of GLP-1 and agents that inhibit the degradation of GLP-1 (7–36) are available as treatments for T2D. Enhancing endogenous GLP-1 production/secretion by direct stimulation of GLP-1 secretion, and promotion of growth and viability, of GLP-1-producing cells may be a novel and more physiological option in incretin-based diabetes therapy. However, this option has not yet been explored to any appreciable extent.

Considering the link between obesity and T2D, reduced GLP-1 secretion in obesity and T2D, as well as the fact that high levels of fatty acids induce apoptosis in a number of different cell types, we investigated the mechanisms by which hyperlipidemia could result in increased apoptosis, and thus reduced L-cell mass and GLP-1 secretory capacity, using an *in vitro* model.

For this model, palmitate was used to simulate high concentrations of FFAs relevant to T2D. Our previous results [6] demonstrate that palmitate induces apoptosis of GLP-1-secreting cells after long term treatment. We could also determine a lipoprotective role for metformin. Being the most widely prescribed anti-diabetic agent in the world, metformin reduces blood glucose primarily by reduced hepatic gluconeogenesis. However, interestingly in the light of our findings, metformin treatment also increases levels of circulating GLP-1 in obese patients with or without T2D [7].

Increased fatty acid oxidation has been reported to increase ROS (reactive oxygen species) production, mediating cell damage and lipotoxicity in β-cells [8,9]. However, the existence of lipotoxicity also in GLP-1-secreting cells is a novel concept and the mechanisms mediating such an effect are unknown.

In the current study, we sought to determine the molecular mechanisms mediating lipotoxicity, and metformin-induced lipoprotection, in GLP-1 secreting-cells. This in order to identify potential molecular targets for increasing endogenous GLP-1 secretion through enhanced viability of GLP-1-secreting cells in diabetic hyperlipidemia and obesity.

2. Materials and methods

2.1. Cell culture and in vitro exposure

The GLP-1-secreting GLUTag cell line (source: glucagon-producing enteroendocrine cell tumor that arose in transgenic mice generated on an out-bred CD-1 background) [10], graciously donated by Dr. Neil Portwood at Karolinska Institutet, Solna, Sweden, and originally from Dr. Daniel J. Drucker, Mount Sinai Hospital, Samuel Lunenfeld Research Institute, University of Toronto, Canada, was cultured and plated 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 media was supplemented with 2% FBS (Sigma–Aldrich), 5.5 mM glucose, 10,000 U/ml penicillin and 10 mg/ml streptomycin sulfate (Invitrogen, Inc.), in addition to 0.44% bovine serum albumin (BSA, fatty acid free) (Sigma–Aldrich). Prior to exposure to Palmitate in Palmitate exposure media, cell were washed twice with 2% FBS, 5.5 mM glucose media. 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 was purchased from Sigma–Aldrich. p38 inhibitor SB203580 and Trolox were purchased from Santa Cruz Biotechnology Inc.

2.2. 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 Bio-Rad DC protein assay (method of Lowry [11], using BSA as a standard [Bio-Rad Laboratories, Hercules, CA]).

2.3. 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) prior to treatment with 0.125 mM palmitate in the presence or absence of 10 μM p38 inhibitor (SB203580) or 1 mM Trolox in 2% FBS and 5.5 mM glucose for an additional 24 or 48 h. Caspase-3 activity assay kit (Cell Signaling Technology, Inc., Danvers, MA) was used 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.

2.4. ROS measurement

GLUTag cells were plated (at a density of 300,000 cells/ml) and grown in 6-well plates for 24 h. Cells were then washed twice with low serum medium (2% FBS, 5.5 mM glucose) prior to treatment with 0.125 mM palmitate and/or 2 mM metformin in 2% FBS and 5.5 mM glucose for an additional 24 h. The Image-iTTM LIVE Green reactive oxygen species (ROS) Detection Kit (Invitrogen, Inc.) used provides the key reagents necessary for the detection of ROS in live cells. The assay is based on 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a reliable fluorogenic marker for ROS in live cells.

2.5. Western blot analysis

GLUTag cellular protein was extracted following culture w/wo 0.125 mM palmitate and/or 2 mM metformin 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 PBS-T; primary (over-night) and secondary (1 h) antibody incubations were performed in the same buffer, with three 10 min washes in PBS-T intervening. Anti-phospho-p38 and anti-p38 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA).

Horseradish peroxidase-conjugated secondary antibodies (1:5000) (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 α-tubulin.

2.6. Statistical analysis

Comparisons between groups, treatments and time were made by a one-way ANOVA for repeated measures. 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.

3. Results

3.1. ROS production is increased in response to palmitate and addition of the antioxidant Trolox prevents the palmitate-induced caspase-3 activation

An increased fatty acid oxidation resulting in increased ROS production has been reported to mediate the effects of palmitate in other cell systems [8,9]. Thus, we hypothesized that palmitate-induced lipotoxicity may be mediated by an increased fatty acid oxidation and ROS production. Consequently, we determined ROS production following 24 h palmitate exposure. Our results demonstrate that 0.125 mM palmitate significantly increase ROS production (Fig 1A). To determine the role of the increased ROS production in palmitate induced caspase-3 activation, we determined the effect of palmitate on caspase-3 activation in the presence or absence of co-incubation with the vitamin E-derived antioxidant Trolox. Our results demonstrate that Trolox does not alter basal caspase-3 activity but significantly attenuates the palmitate induced caspase-3 activation (Fig 1B).

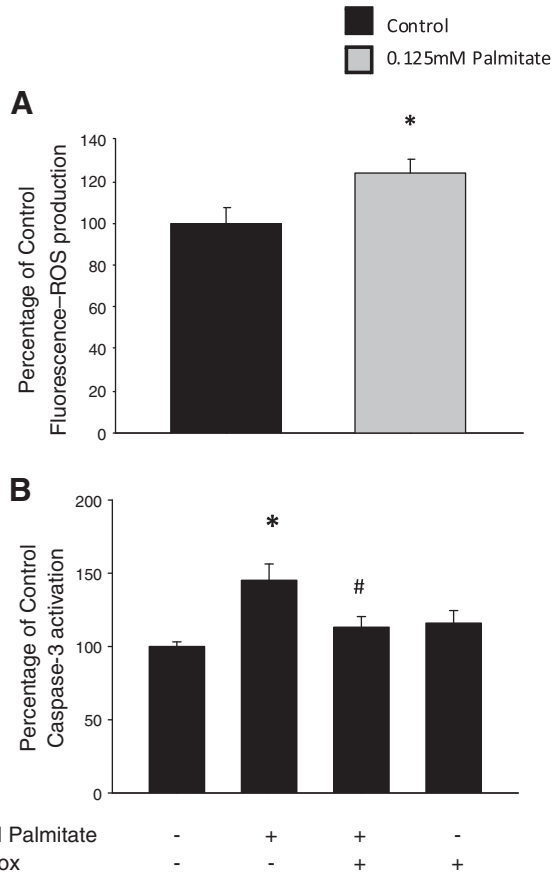

Fig. 1. ROS production is increased in response to palmitate and addition of the antioxidant Trolox prevents the palmitate-induced caspase-3 activation. 0.125 mM palmitate significantly increases ROS production in GLP-1-secreting cells after 24 h as measured by fluorescence of the ROS sensitive probe carboxy-H_2DCFDA (A). Co-incubation with 1 mM of the vitamin E-derived antioxidant Trolox inhibits increased caspase-3 activity in response to 0.125 mM palmitate (B) ($n = 3$). Bars represent mean ± SEM. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared with palmitate-treated cells.

3.2. The ROS sensitive MKK4-JNK and MKK3/6-p38 pathways mediate caspase-3 activation in GLP-1 secreting cells in response to simulated hyperlipidemia

ROS is a well-known activator of apoptosis signal-regulating kinase 1 (ASK1), by providing oxidating conditions, where the inhibitory association of ASK1 with thioredoxin is dissolved. Subsequent ASK1 activation initiates a signaling cascade with activation of the MAPKK 4 (MKK4)-JNK and MKK3/6-p38 pathways but not the MAP/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) [12]. These signaling events can be compared to our previous findings of unaltered ERK phosphorylation, but increased JNK phosphorylation in response to 0.125 mM palmitate, where pre-incubation with JNK inhibitor SP600125 reduces palmitate induced caspase-3 activity [6]. In the present study we determined the role of the other MAPKK regulated by ROS and ASK1 and phosphorylated by palmitate, MKK3/6-p38. Our results show that palmitate significantly increases the phosphorylation of p38 (Fig. 2A and B). Further, a 1 h pre-incubation with the p38 inhibitor (SB203580) significantly reduces palmitate-induced caspase-3 activity (Fig. 2C).

3.3. Metformin increases ROS production, while reducing the expression of p38 and mediating lipoprotection

We have previously reported a lipoprotective effect in response to 2 mM metformin, where a pre-incubation with metformin significantly attenuates palmitate induced caspase-3 activity and DNA fragmentation [6]. Subsequently, we aimed to determine if pre-incubation with 2 mM metformin could alter palmitate-induced ROS production and/or the activity of the p38 signaling pathway. We demonstrate here that 2 mM metformin further increases palmitate-induced ROS production (Fig. 3A). However, while no significant effect on p38 phosphorylation in response to pre-incubation of palmitate with 2 mM metformin was detected, the expression of p38 was significantly reduced (Fig. 3B and C).

4. Discussion

In this study, we provide evidence for increased ROS production as a mediator of lipotoxicity in GLP-1-secreting cells *in vitro*. We show that simulated hyperlipidemia increases ROS production and phosphorylates p38 in the same cell system, where addition of antioxidants or inhibition of p38 can effectively reduce lipotoxicity. Further, metformin confers lipoprotection [6] and – despite increased ROS production – reduces the expression of p38 under these lipotoxic conditions.

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. Throughout this study palmitate was used to simulate diabetic hyperlipidemia *in vitro*, as it is the most abundant saturated FFA bound to human serum albumin [15]. We have previously reported lipotoxicity in enteroendocrine GLP-1-secreting L-cells in response to the relevant concentration of palmitate *in vitro* based on determination of viable cells, DNA fragmentation and caspase-3 activity at the end of a 48 h incubation [6]. To

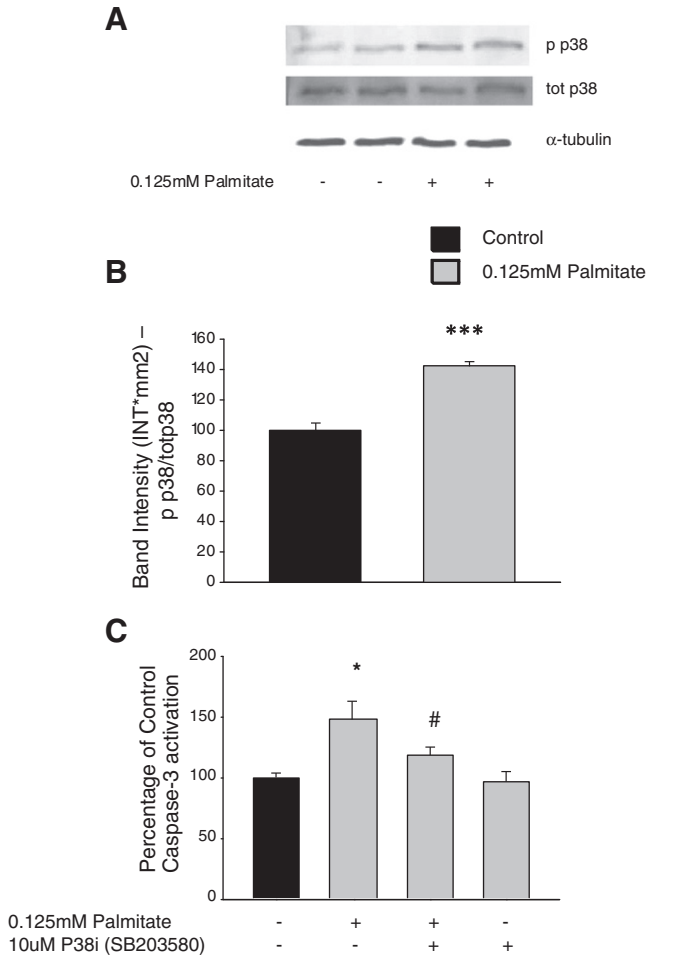

Fig. 2. The ROS sensitive MKK4-JNK and MKK3/6-p38 pathways mediate increased caspase-3 activation in GLP-1-secreting cells in response to simulated hyperlipidemia. 0.125 mM palmitate significantly increases phosphorylation of p38 after 8 h. Representative blot (A) and statistical analysis (B). Pre-incubation of 0.125 mM palmitate with the p38 inhibitor (SB203580) significantly attenuates palmitate-induced caspase-3 activity after a 48 h incubation ($n = 3$). Bars represent mean ± SEM. $^{*}p < 0.05$ compared with controls. $^{***}p < 0.001$ compared with controls. $^{\#}p < 0.05$ compared with palmitate-treated cells.

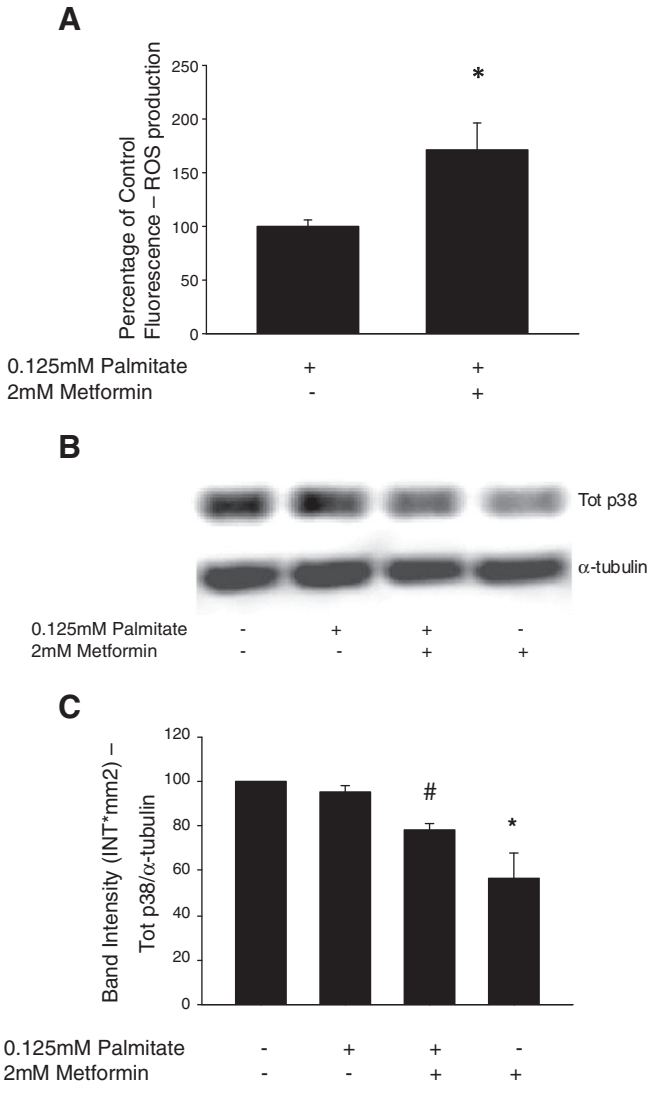

Fig. 3. Metformin increases ROS production, while reducing the expression of p38 and mediating lipoprotection. In the presence of 0.125 mM palmitate, 2 mM metformin significantly increases ROS production after 24 h, as measured by fluorescence of the ROS-sensitive probe carboxy-H_2DCFDA (A). Representative blot (B) and statistical analysis (C) showing that 2 mM metformin downregulates the expression of the p38 protein ($n = 3$). Bars represent mean ± SEM. $^{*}p < 0.05$ compared with controls.

further study the mechanisms of this induced lipotoxicity in the present study, we have used caspase-3 activation in response to palmitate as a measure of lipoapoptosis. As a model for GLP-1 secreting L-cells we used GLUTag cells, a stable immortalized murine enteroendocrine cell line that expresses the proglucagon gene and secretes glucagon-like peptides [10]. GLUTag cells appear quite well differentiated, and recapitulate the responsiveness of primary intestinal L-cell cultures to physiological and pharmacological GLP-1 secretagogues [16,17]. The GLUTag cell line is one of the best models for studying the L-cells, since native L-cells are very scarce and dispersed along the gastrointestinal tract as single cells, and cannot be isolated to provide homogenous L-cell cultures.

When investigating the molecular mechanisms underlying lipotoxicity induced by simulated hyperlipidemia, we first studied ROS production in the presence/absence of simulated hyperlipidemia, as increased ROS production in response to palmitate has been reported to mediate cell damage and apoptosis in insulin-producing β-cells [8,9]. In addition to observing an increase in ROS production, we could effectively reduce lipotoxicity in response to palmitate using the antioxidant and ROS scavenger Trolox. With an increased ROS production, it is expected to see the observed activation of ROS sensitive pathways such as ASK1 and downstream MKK such as JNK – as previously reported [6] – and p38 phosphorylation. It appears that JNK and p38 may together be the mediators of lipoapoptosis downstream of increased ROS production as the JNK inhibitor SP600125 significantly attenuated palmitate-induced caspase-3 activity by ~20% [6] and, as the present study reveals, p38 inhibition results in an ~80% attenuation of caspase-3 activity. However, pre-incubation of palmitate with both inhibitors would be necessary before concluding that these are additive effects, and that inhibiting both kinases will completely block palmitate-induced lipotoxicity. In addition, a future goal is to inhibit fatty acid oxidation and to determine ROS production and the effect on JNK and p38 phosphorylation after addition of ROS scavengers, such as SOD and/or Trolox.

Surprisingly, we did not find a decrease in ROS production by metformin as observed in rat pancreatic islets and β-cells [18]. The lipoprotective effects of metformin have, in other *in vitro* cell studies, been reported to result from protection against oxidative cell injury by induction of a metabolic stress response with stabilization of the mitochondrion where its oxidative capacity is increased [19]. The metformin-induced increase in ROS production,

in conjunction with reduced expression of the ROS sensitive MAP-KK p38 shown in the present study, indicates reduced activation of the p38 pathway despite increased ROS production. This may demonstrate a metformin lipoprotective effect dependent on increased oxidative capacity also in GLP-1-secreting cells. The concentration of metformin (2 mM) used in this *in vitro* study is supratherapeutic. However, it is important to consider that cells in culture are grown in an environment of overabundant nutrients. This is an inherent limitation of *in vitro* studies using cell lines and may be why higher concentrations of drugs are needed to see the effects typically seen in patients. In addition, metformin accumulates in tissues at higher concentrations than in blood [20], and as the L-cells directly face the intestinal lumen, they may locally be exposed to very high concentrations of metformin.

The protection of GLP-1-secreting cells against lipoapoptosis by metformin may provide an explanation to the increased plasma GLP-1 levels seen in diabetic patients on chronic metformin treatment [7], as protecting these cells from apoptosis induced by high levels of FFAs could contribute to an increased L-cell mass and increased GLP-1 secretion. Consequently, the molecular mechanisms mediating lipoapoptosis/lipoprotection of GLP-1-secreting cells become important in diabetes research.

However, whether 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. *In vivo* studies using mice that have developed hyperglycemia and impaired glucose tolerance following 10 weeks on a high fat diet are under way in our laboratory. Immunohistological evaluation of L-cell mass, growth and apoptosis in these mice as compared to healthy controls may provide indications as to the clinical relevance of this lipotoxicity.

In conclusion, more data is needed to define the exact mechanism of metformin-induced lipoprotection of GLP-1-secreting cells, and to determine the role of the most well known target for metformin action, AMP-activated protein kinase (AMPK), in this lipoprotection. However, the present study provides novel information on the underlying molecular mechanisms of lipotoxicity in this cell type. Specifically, we demonstrate here that lipotoxicity in GLP-1-secreting cells *in vitro* can be counteracted by ROS scavengers. This indicates that it results from increased ROS production, activating the p38 pathway, where signaling downstream of p38 mediates increased caspase-3 activity and apoptosis – findings suggesting that increased fatty acid oxidation mediates lipotoxicity in these cells. Further, we show that metformin downregulates the MKK3/6-p38 pathway by significantly reducing the expression of p38, and exerts a lipoprotective effect in conjunction with increased ROS production, which suggests an increased oxidative capacity in the presence of metformin. Insight into the mechanisms channeling excess fatty acids to particular metabolic fates in these cells may contribute to the development of more effective therapies for T2D based on increased viability of GLP-1-secreting cells and increased endogenous secretion of the incretin.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contribution

Camilla Kappe: Performed and designed the experiments, contributed to the research plan and discussions, wrote the manuscript, performed analysis of data, acquired and processed images and figures. *Jens Juul Holst*: Provided expertise on GLP-1. Participated in manuscript preparation. *Qimin Zhang*: Conceived the research plan, participated in discussions and manuscript preparation. Åke Sjöholm: Conceived the hypotheses and research plan, participated in discussions, provided expertise in diabetes and contributed to manuscript preparation and editing.

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