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The antiepileptic drug topiramate preserves metabolism-secretion coupling in insulin secreting cells chronically exposed to the fatty acid oleate

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Abbreviations:
CPT-1, carnitine palmitoyl transferase
FAT, fatty acid translocase
FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
GSIS, glucose stimulated insulin secretion
PPARα, peroxisome proliferator-activated receptor α
TPM, topiramate

ABSTRACT

Topiramate (Topamax), primarily prescribed against epilepsy, was reported to reduce body weight and to ameliorate glycemic control in obese patients with diabetes. In rodent models of obesity and diabetes, topiramate treatment counteracts hyperglycemia and increases insulin levels upon glucose tolerance test. These observations suggest that topiramate might exert direct action on insulin secreting cells, in particular regarding obesity associated β-cell dysfunction. In this study, INS-1E β-cells were exposed for 3 days to the fatty acid oleate (0.4 mM) and concomitantly treated with therapeutic concentrations of topiramate before measurements of insulin secretion and metabolic parameters. In healthy cells, topiramate had no acute or chronic effects on insulin release. Exposure of INS-1E cells to oleate for 3 days increased insulin release at basal 2.5 mM glucose and blunted the response to stimulatory glucose concentration (15 mM). Such lipotoxic effects were associated with impaired mitochondrial function, as evidenced by partial loss of resting mitochondrial membrane potential and reduced hyperpolarization in response to glucose. Oil-red-O staining and triglyceride measurements revealed lipid accumulation in oleate treated cells. Topiramate treatment counteracted oleate-induced lipid load and partially protected against mitochondrial membrane dysfunction. In particular, topiramate restored glucose stimulated insulin secretion, essentially by maintaining low insulin release at basal glucose. Topiramate increased expression of the nutrient sensor PPARα and of the mitochondrial fatty acid carrier CPT-1, correlating with enhancement of β-oxidation rate. The data demonstrate that a drug originally used as mood stabilizer exerts a direct action on β-cells, protecting against lipid-induced dysfunction.

1. Introduction

Topiramate (TPM) is a sulphamate-substitute monosaccharide currently indicated in the treatment of epilepsy. TPM is considered to produce its antiepileptic effects through several mechanisms including: (i) enhancement of GABAergic activity; (ii) inhibition of AMPA/Kainate type glutamate receptors; (iii) inhibition of sodium channels, L- and N-type

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calcium channels and the isoenzymes of carbonic anhydrase CA-III and CA-IV [1]. TPM, structurally considered as an anticonvulsant, is being evaluated for other neurological pathologies such as migraine, neuropathic pain, essential tremor, and also for psychiatric conditions such as bipolar disorders, bulimia, post-traumatic stress disorders and schizophrenia [2].

Several clinical studies showed that treatment with TPM is associated with significant weight loss of the patients, in contrast with the effects of traditional mood stabilizers generally leading to body weight gain [3–6]. In a recent study following 307 obese subjects with type 2 diabetes over 1 year, TPM was effective for weight reduction and improvement in glycemic control [7]. The mechanisms at the basis of these effects have not been elucidated. Chronic treatment with TPM in Zucker diabetic fatty (ZDF) rats and db/db mice improves hyperglycemia, an effect associated with elevated insulin levels upon oral glucose tolerance test [8]. Several experiments in rats demonstrated that TPM reduces energy deposition with a gradual and persistent effect during the treatment [9]. Inhibition of weight gain is evident in lean and obese subjects, although changes in food intake are more pronounced in the latter group and reduction of body fat accounts for the whole weight reduction [10,11].

TPM has been reported to inhibit the activity of lipoprotein lipase in different white adipose tissue depots, accounting for the reduction in fat deposition [11]. The drug also inhibits lipoprotein lipase in brown adipose tissue and skeletal muscle, indicating its capacity to enhance regulatory thermogenesis and substrate oxidation [11]. TPM was shown to decrease glucose and triglycerides levels in blood [9,12]. This effect is in part explained by the capacity of TPM of improving insulin sensitivity in adipose tissue, liver, and muscle in obese rats due to a selective insulin sensitization of the adipose tissue independent of weight loss [13].

One aspect that remains uninvestigated is the potential direct effect of TPM on pancreatic β-cells. Published data suggest that amelioration of β-cell function, in models associated with lipid and/or glucotoxicity, might account for the effects of TPM [8]. In order to understand the mechanisms responsible for the lowering of blood glucose, an action of TPM on pancreatic β-cells should be investigated. The present work shows that TPM, used in the range of therapeutic concentrations of 1–10 μM [14], protects metabolism-secretion coupling in insulin secreting cells against chronic lipid exposure.

2. Materials and methods

2.1. Cell culture

INS-1E β-cells were used as a well-differentiated clone derived from rat insulinoma INS-1 cells [15]. INS-1E were cultured in humidified atmosphere containing 5% CO_2 in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10 mM Hepes, 5% (v/v) Fetal Calf Serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol.

2.2. Cell treatment

INS-1E cells were seeded in 24-well plates in RPMI-1640. After 3 days of culture, cells were supplemented with 0.4 mM oleate (stock solution: 10 mM oleate (Sigma–Aldrich, Germany) dissolved in 12.5% fatty acids free BSA (Sigma–Aldrich)) and with 0.4 mM oleate plus 1, 3 and 10 μM TPM (stock solution: 10 mM TPM (Johnson & Johnson, USA) dissolved in bi-distilled water). Where indicated, cells were treated for 3 and 6 days. For pre-treatment experiments, cells were supplemented with 0.4 mM oleate for 3 days, followed by 3 days of 10 μM TPM addition. For the acute treatment, 10 μM TPM were added to INS-1E cells 10 min after glucose stimulation.

2.3. Mitochondrial membrane potential ($\Delta\Psi_m$) measurement

Cells were cultured in 24-well plates and treated as described above. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured as described [15]. In brief, INS-1E β-cells were incubated with 10 μg/ml Rhodamine-123 (Molecular Probes, Eugene, OR) for 20 min, at 37 °C in KRBH medium (Krebs–Ringer bicarbonate-HEPES buffer: 129 mmol/l NaCl, 5 mmol/l NaHCO3, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 10 mmol/l HEPES, and 1 mmol/l $CaCl_2$ at pH 7.4, with 0.1% BSA). The $\Delta\Psi_m$ was monitored with excitation and emission filters set at 485 and 520 nm respectively. Glucose 15 mM (on top of basal 2.5 mM) and 1 μM of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, Sigma–Aldrich) were added at the indicated times.

2.4. Insulin secretion assay

INS-1E β-cells were cultured in 24-well plates and treated as described above. In order to measure INS-1E secretory responses, cells were maintained for 2 h in glucose-free RPMI medium and then incubated for 30 min in KRBH containing the indicated glucose concentrations or 30 mM KCl. At the end of the incubation period, supernatants were collected for quantification of insulin concentrations. Cells were extracted with acid-EtOH in order to measure their insulin contents. Insulin levels were determined by radioimmunoassay (Linco, St. Charles, MO) using rat insulin as standard. Insulin secretion was expressed as the percentage of insulin release normalized to cellular insulin content (%content) in order to reflect basal and stimulated insulin release. The secretory response to glucose was expressed as the glucose stimulated insulin secretion (GSIS).

2.5. Mitochondrial morphology

INS-1E cells were plated in a 24-well plate on polyornithine-coated glass coverslips at the density of 50,000 cells/ml and treated with oleate and TPM for 3 days. Mitochondrial morphology was revealed by MitoTracker (Molecular Probes) staining. Cells were incubated with MitoTracker 100 nM at 37 °C for 25 min, fixed in 4% paraformaldehyde and washed extensively in phosphate-buffered saline before mounting on glass slides. Cells were viewed using a confocal laser scanning 410 microscope (Carl Zeiss, Gottingen, Germany).

2.6. Oil-Red-O staining

To estimate lipid load in INS-1E cells upon exposure to oleate and TPM, cells were subjected to Oil-Red-O staining. After specific treatments as indicated, cells were fixed with 4% paraformaldehyde and stained in Oil-Red-O (stock solution: 5 g/l dissolved in 60% triethyl-phosphate; working solution: 60:40 Oil-Red-O stock: distilled water) for 1 h. Photographs were taken on Axiovert 25 microscope (Carl Zeiss).

2.7. Triglyceride content

INS-1E were cultured in 12-well plates and treated with 0.4 mM oleate and 3 μM TPM for 3 days. Triglycerides were extracted following Folch's method [16]. In brief, cells were collected in PBS and lysed in MeOH. The lipid phase was separated by adding chloroform and water to the lysate. For the quantification of triglyceride content, samples were mixed with Triglyceride reagent (Roche Diagnostic, Germany) and measured spectrophotometrically at 500 nm.

2.8. β-oxidation rate

INS-1E cells were seeded in 25 cm^2-flasks and after 2 days treated (10 μM TPM) or not (controls) for the last 3 days of culture. Then, cells were pre-incubated for 30 min in glucose free RPMI-1640 medium supplemented with 0.5 mM L-carnitine. β-oxidation was next stimulated for 4 h at 37 °C in KRBH containing 18 mM [1-^{14}C]oleate (final specific activity of 13.9 GBq/mmol). The reaction was stopped by the addition of 6 M HCl and the released $^{14}CO_2$ was quantified in scintillation liquid Lumagel Plus (LUMAC*LSC, Groningen, The Netherlands) using LKB Wallac 1217 Rackbeta counter (Turku, Finland). Experiments were performed in triplicates and data normalized for total cellular proteins.

2.9. Quantitative RT-PCR

INS-1E β-cells were cultured in 10 cm dishes and treated with 0.4 mM oleate and 10 μM TPM. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) and 2 μg were converted into cDNA [17]. Primers for peroxisome proliferator-activated receptor α (PPARα), (sense 5′-TGGCGTTCGCAGCTGTT-3′, antisense 5′-ACCCAGCGTCGCTTCAGTT-3′), carnitine palmitoyl transferase (CPT-1), (sense 5′-CGTGACGTTGGACGAATCG-3′, antisense 5′-CTTCCATGCAGCAGGGATT-3′) and FAT/CD36 (sense 5′-CATCGGCGATGAGAAAGCA-3′, antisense 5′-AACCAGGCCCAGGAGTA-3′) were designed using the Primer Express Software (Applera Europe). Real Time PCR was performed using an ABI 7000 Sequence Detection System (Applera), and PCR products were quantified fluorometrically using the SYBR Green Core Reagent kit. The values obtained were normalized to the values of the reference mRNA β-actin.

2.10. Data analysis

Secretion data, mitochondrial membrane potential quantifications, and cellular lipid levels were analyzed using the SPSS 14.0 statistical package. Specifically, statistical tests were calculated using one-way ANOVA. Pair wise testing with a post hoc multiple comparison procedure (Tukey–Kramer) was used when the group comparison indicated significant group differences. Expression data were analyzed using two-tailed Student's *t*-test. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of TPM on healthy INS-1E cells

Glucose stimulation induces cellular metabolic responses in β-cells leading to mitochondrial activation and subsequent insulin exocytosis. We tested putative direct action of TPM on key parameters of metabolism-secretion coupling in INS-1E β-cells. In control cells, stimulation with 7.5 and 15 mM glucose increased insulin release, 2.9-fold ($P < 0.05$) and 6.6-fold ($P < 0.05$) respectively, compared to basal release at 2.5 mM glucose. Non-nutrient stimulation with 30 mM KCl, inducing cytosolic calcium rise, stimulated insulin secretion 3.8-fold ($P < 0.01$) compared to basal. The secretory response to glucose and KCl were not modified by simultaneous exposure to TPM up to 10 μM (Fig. 1A). Culture of INS-1E cells in the presence of 10 μM TPM for 3 days was without effects on glucose stimulated insulin secretion (Fig. 1B).

As a key metabolic parameter, we measured mitochondrial membrane potential in INS-1E cells. As expected, the mitochondrial membrane was hyperpolarized upon glucose elevation (from 2.5 to 15 mM) resulting in a drop in the rhodamine-123 signal of 10.5 ± 2.3% ($n = 3$), calculated 6 min after glucose stimulation. Acute exposure of INS-1E cells to 10 μM TPM resulted in similar glucose responses (11.3 ± 2.9%) and preincubation of the cells with 10 μM TPM for 10 min did not modified glucose-induced hyperpolarization (10.4 ± 0.1%). The data show that neither acute exposure nor chronic 3-day treatments with 10 μM TPM did modify the glucose response compared to control cells (Fig. 2A and B).

3.2. Effects of oleate and TPM on insulin secretion

We next tested the effects of TPM in cells chronically exposed to lipids, by measuring the secretory responses of INS-1E cells over a 30 min stimulation period following 3-day culture in the presence of 0.4 mM oleate with and without 0.1, 1, 10 and 100 μM TPM. Fig. 3A shows the effects induced by oleate treatment on GSIS. Chronic exposure to oleate increased basal (2.5 mM glucose) insulin release (3.9-fold, $P < 0.001$). Consequently, the secretory response to stimulatory glucose concentration (15 mM) observed in control cells (5.3-fold) was inhibited in oleate treated cells. This demonstrates the lipotoxic effects of chronic exposure to fatty acids on GSIS in INS-1E cells. Cellular insulin contents were not different after the 3-day culture in the presence of normal medium compared to cells exposed to 0.4 mM oleate without and with 1 and 10 μM TPM (1.98 ± 0.59, 1.65 ± 0.34, 1.78 ± 0.51, 1.99 ± 0.06 μg insulin per well, respectively). These insulin contents are in the range expected for INS-1E cells [15]. It should be noticed that the sensitivity of primary islets in terms of duration of exposure to oleate might be different (data not shown), as corresponding

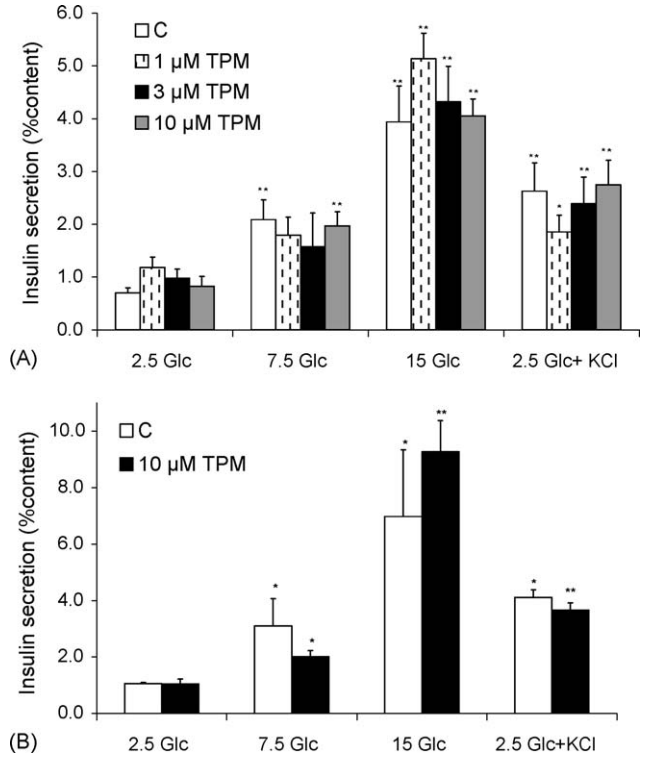

Fig. 1 – Effects of acute and chronic treatments with TPM on insulin secretion in INS-1E cells. (A) INS-1E cells, cultured in 24-wells plates, were challenged for 30 min with glucose at 2.5 mM (2.5 Glc), 7.5 mM (7.5 Glc), and 15 mM (15 Glc). Insulin release was also stimulated with 30 mM KCl at basal 2.5 mM glucose (2.5 Glc + KCl). Acute treatment with TPM was tested by addition of the drug at the indicated concentrations for the 30 min stimulation period only. (B) Chronic treatment with TPM (10 μM) was tested on INS-1E cells for 3 days. At the end of the culture period, in the absence or presence of TPM, cells were stimulated for 30 min with glucose (2.5, 7.5, and 15 mM) and KCl (30 mM). Values are means + S.D. of one representative out of five different experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TPM concentration-matched basal secretion (2.5 Glc).

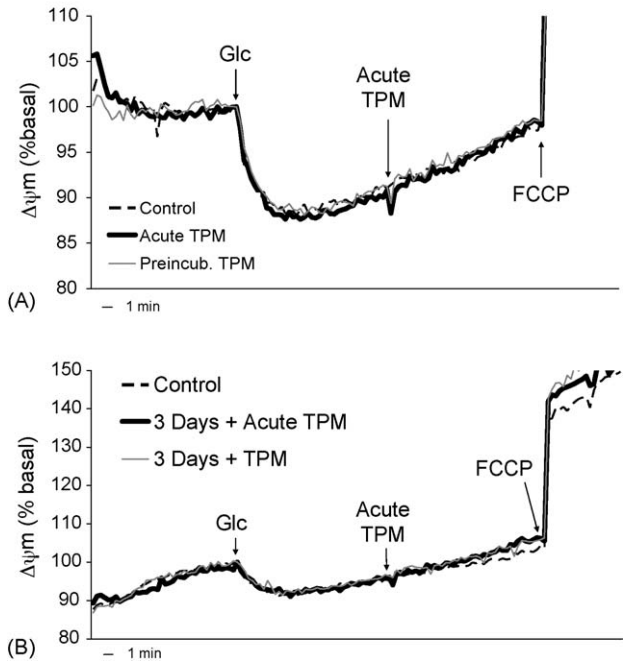

Fig. 2 – Effects of TPM on mitochondrial membrane potential ($\Delta\Psi_m$) in INS-1E cells. (A) Hyperpolarization of $\Delta\Psi_m$ was induced in INS-1E cells by elevating glucose from basal 2.5–15 mM (see arrow, Glc). After 10 min of glucose stimulation, TPM (10 μM) was added to cells as an acute exposure. Preincubation with 10 μM TPM for 10 min (Preincub.) before glucose stimulation was also tested. (B) Effects of TPM treatment for 3 days of culture before measurements of mitochondrial membrane potential in INS-1E cells. Hyperpolarization of $\Delta\Psi_m$ was induced by glucose 15 mM (Glc) and further acute TPM exposure (Acute TPM, 10 μM) was tested 10 min after glucose stimulation. Complete depolarization of the mitochondria was induced as control at the end of each recording by the addition of 1 μM of the protonophore FCCP. Traces are representative of at least three independent experiments performed in triplicate.

β-cells are wrapped up in a protective structure as opposed to monolayer used in the present study.

A dose response of TPM was tested by culturing cells with oleate plus 0.1–100 μM of the drug. Fig. 3B shows the effect of TPM treatment on the secretory response to 15 mM glucose compared to basal release, i.e., GSIS. The dose response exhibited a bell-shape effect with partial restoration of GSIS at TPM concentrations 1 μM (2.0-fold, $P < 0.005$) and 10 μM (1.7-fold, $P < 0.05$).

3.3. *Effects of oleate and TPM on mitochondrial function*

We next investigated the effects of TPM and oleate treatments on mitochondrial membrane potential. Three days of culture in the presence of oleate impaired glucose-induced mitochondrial hyperpolarization (Fig. 4A). After 6 min of glucose stimulation, rhodamine-123 signal was lowered by 12.7 ± 5.4% in control cells, thereby reflecting efficient mitochondrial hyperpolarization. In cells exposed for 3 days to 0.4 mM oleate, the glucose response was attenuated (7.8 ± 2.2%, $P < 0.05$ versus controls). Simultaneous treatment with TPM partially prevented the lipotoxic effects observed on the respiratory chain function (Fig. 4A), as evidenced by the amplitude of mitochondrial hyperpolarization (11.0 ± 2.2%, $P < 0.02$ versus oleate). Similar effects were observed when cells were pre-treated with oleate for 3 days before TPM supplementation for another 3-day period (data not shown). These results indicate that TPM can also reverse the deleterious effects associated with oleate exposure. Disruption of mitochondrial membrane potential by the addition of the protonophore FCCP resulted in complete depolarization of the mitochondria, thereby reflecting the amplitude of total mitochondrial membrane potential. Such measurements (Fig. 4B) revealed that mitochondria were partially depolarized following oleate exposure (−12%, $P < 0.01$).

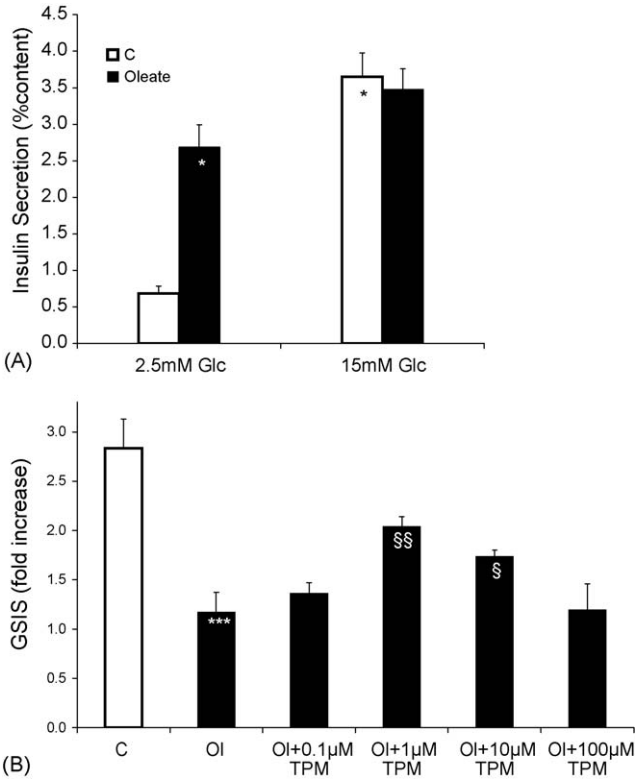

Fig. 3 – Effects of TPM on insulin secretion in INS-1E cells cultured with fatty acids. INS-1E cells were seeded in 24-well plates and cultured for the last 3 days preceding the experiment in the presence of 0.4 mM oleate in the absence or presence of 0.1, 1, 10, and 100 μM TPM. At the end of the culture period, insulin secretion was measured from cells stimulated for 30 min with 15 mM glucose (Glc) compared to basal release at 2.5 mM Glc. (A) Insulin secretion from cells cultured in control conditions (C) and following 3 days of exposure to 0.4 mM oleate (Oleate). $^{*}P < 0.001$ vs. control at 2.5 mM Glc. (B) Secretory responses to 15 mM glucose compared to basal release (GSIS) in INS-1E cells tested at the end of a culture period of 3 days in control conditions (C) or in the presence of 0.4 mM oleate (OI) supplemented or not with TPM as indicated. $^{***}P < 0.001$ vs. control; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs. oleate. Results are representative of at least three independent experiments performed in triplicate.

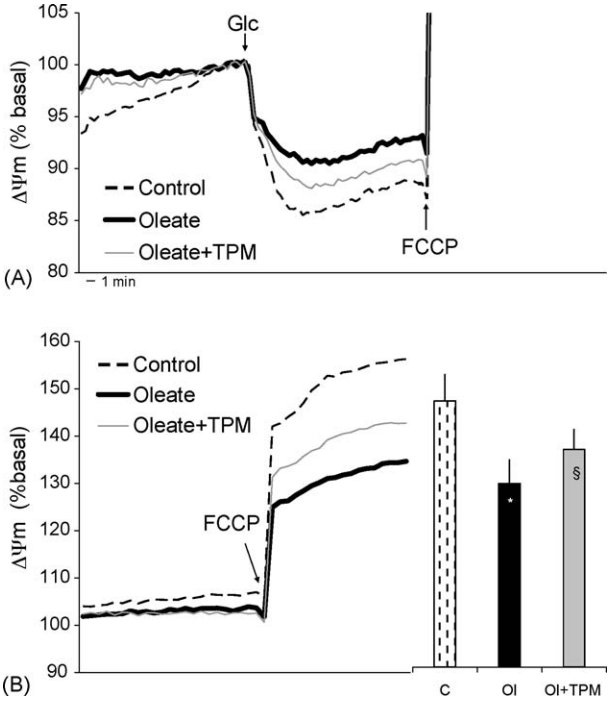

Fig. 4 – Effects of TPM on mitochondrial membrane potential ($\Delta\Psi_m$) in INS-1E cells cultured with fatty acids. INS-1E cells were seeded in 24-wells plates and cultured for the last 3 days in the presence of 0.4 mM oleate in the absence or presence of 10 μM TPM. (A) Hyperpolarization of $\Delta\Psi_m$ was induced in INS-1E cells by elevating glucose from basal 2.5–15 mM (see arrow, Glc). Control cells were cultured in normal conditions. (B) Complete depolarization of the mitochondria was induced in cells incubated at basal 2.5 mM glucose by the addition of the protonophore FCCP (1 μM). Quantification of total $\Delta\Psi_m$ was estimated as amplitude of complete depolarization following dissipation of the proton gradient (shown as bars in B). Traces shown in A and B representative traces of 5–7 experiments and bar-graphs in B are means ± S.D. of three independent experiments ($^{*}P < 0.01$ vs. control; $^{\S}P < 0.05$ vs. oleate).

TPM treatment prevented ($P < 0.05$) part of the deleterious effects associated with oleate.

Fig. 5 shows mitochondrial morphology of INS-1E cells compared to cells cultured in the presence of oleate and oleate plus TPM. After 3 days of oleate exposure, cells displayed condensed mitochondria with a globular pattern, also indicative of reduced membrane potential. TPM treatments (1 and 10 μM) prevented oleate-induced alteration of mitochondrial morphology (Fig. 5C and D).

3.4. *Effects of TPM on lipid metabolism in INS-1E cells*

We next investigated lipid contents of INS-1E cells exposed to oleate and TPM. Cells were cultured with oleate and TPM for 3 or 6 days as described above. Oil-Red-O staining on INS-1E treated with oleate exhibited strong red signal (Fig. 6), indicating important lipid accumulation compared to control cells. Cell morphology was affected, presenting smaller and rounded cellular bodies. INS-1E cells treated with TPM were partially preserved both from lipid accumulation and morphological alterations. INS-1E β-cells cultured with oleate for 3 days before TPM treatment were also partially protected from lipid accumulation (Fig. 6E and F).

As Oil-Red-O staining is only indicative of cellular fat content, lipids were directly quantified by measurements of triglyceride concentrations in INS-1E cells (Fig. 6G). Exposure to oleate for 3 days increased triglyceride contents (+133%, $P < 0.01$), an effect counteracted by TPM treatment (−39%, $P < 0.05$).

Intracellular lipid consumption capacity was assessed by measuring β-oxidation rate in INS-1E cells following a 3-day treatment with 10 μM TPM. Cells were not previously cultured

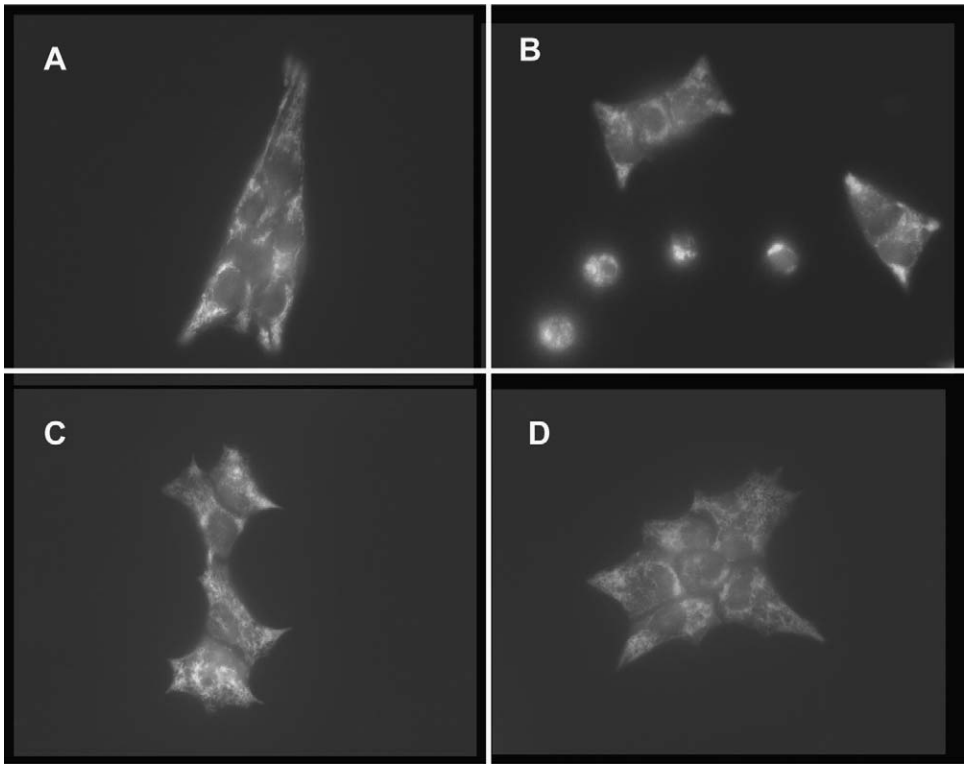

Fig. 5 – Effects of TPM on mitochondrial morphology in INS-1E cells exposed to fatty acids. INS-1E cells were cultured for the last 3 days in the presence of 0.4 mM oleate in the absence or presence of TPM (1 and 10 μM) before Mitrotracker staining and fixation. (A) Control cells exhibiting elongated mitochondria; (B) oleate treated cells; (C) cells exposed to oleate and 1 μM TPM; (D) cells exposed to oleate and 10 μM TPM. The results shown are representative of three independent experiments.

with oleate in order to avoid differences in intracellular oleate concentrations at the time of the assay resulting in group-specific dilutions of the specific activity. In control cells, oxidation rate of 18 mM [1-^{14}C]oleate to $^{14}CO_2$ was 8.93 ± 0.22 nmol/mg prot per hour (n = 3 in one representative out of 3 independent experiments). In TPM treated cells, lipid oxidation was 11.08 ± 0.24 nmol/mg prot per hour, corresponding to a 24% increase versus non-treated cells ($P < 0.001$). These measurements reflect the enhanced capacity of lipid consumption conferred by TPM treatment.

3.5. *Effects of oleate and TPM on metabolic gene expression*

In order to investigate the mechanisms underlying the protective effects of TPM on INS-1E β-cells exposed to fatty acids, we measured the expression of genes involved in lipid and carbohydrate metabolism by quantitative RT-PCR (Fig. 7). We isolated mRNA from INS-1E cells treated for 3 days with 0.4 mM oleate and 10 μM TPM. Cells treated with oleate displayed decreased expression of the lipid transcription factor PPARα (Fig. 7A) in 6 out of 9 independent experiments. TPM treatment attenuated the decrease in PPARα expression observed with oleate, as TPM increased PPARα expression 1.74-fold compared to oleate ($P < 0.02$), an effect observed in all the 9 independent experiments. This elevation in PPARα suggests that TPM can promote lipid catabolism in the INS-1E cells. Accordingly, we tested the levels of CPT-1 transcript, as it is involved in fatty acid oxidation and related to PPARα activity. CPT-1 expression increased 2.5-fold ($P < 0.001$) in the presence of oleate. TPM treatment further induced up-regulation of CPT-1, displaying an increment of 1.6-fold ($P = 0.01$) versus cells exposed to oleate only. Moreover, TPM treated cells exhibited increased expression (1.3-fold, $P < 0.05$) of the fatty acid translocase FAT/CD36, whose expression was not affected by lipid load alone.

4. Discussion

TPM is a neurotherapeutic drug currently indicated for the treatment of seizures, bipolar disorders, neuropathic pain and migraine [18]. Recent studies reported an additional activity on body weight loss, increase in energy expenditure and peripheral sensitization to insulin action [3,6]. In rodent diabetic models, TPM was found to increase plasma insulin levels upon glucose load, independently of weight gain or food intake [8]. As pancreatic β-cells are highly sensitive to lipid-induced toxicity [19], these recent data suggest the possibility of direct action of TPM on insulin secreting cells exposed to fatty acids. In this context, we explored putative protective effects that TPM might exhibit on metabolism-secretion coupling in INS-1E β-cells cultured in the presence of oleate, a model of lipid-induced impairment of metabolism secretion coupling [20]. Our results show that, although TPM had no effect in healthy cells, it counteracted the deleterious consequences of lipid exposure.

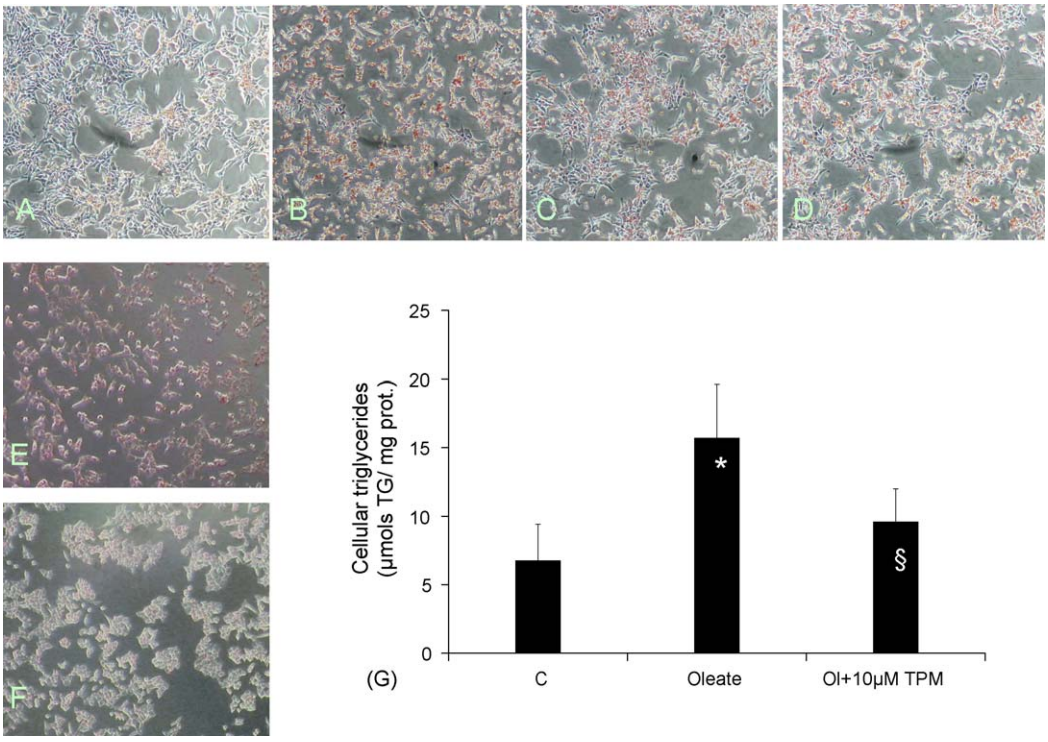

Fig. 6 – Effects of TPM on INS-1E cells morphology and lipid accumulation. INS-1E cells were cultured with 0.4 mM oleate in the absence or presence of TPM before measurements of lipid load. (A–F) Oil-Red-O staining in INS-1E cells showing morphology and indicative of intracellular lipids. Cells were cultured for 3 days without oleate (A), with oleate (B), with oleate plus 1 μM (C) or 10 μM (D) TPM. Cells were also exposed to oleate for 6 days (E) plus 10 μM TPM only for the last 3 days (F). Pictures are representative of five independent experiments. (G) Cellular triglyceride levels measured in INS-1E cells treated for 3 days in normal medium without oleate (C) or with oleate in the absence (Oleate) or presence of 10 μM TPM (Ol + 10 μM TPM). $^{*}P < 0.01$ vs. control; $^{§}P < 0.05$ vs. oleate.

Lipotoxicity is one of the major causes of β-cell dysfunction in type 2 diabetes. Prolonged exposure of β-cells to high levels of fatty acids can cause impairment of metabolic genes expression leading to decreased glucose-stimulated insulin secretion [19,21]. Here, chronic exposure of INS-1E cells to the fatty acid oleate resulted in impaired mitochondrial activation, accumulation of lipids in the cytoplasm, and reduced glucose-induced insulin secretion. Simultaneous treatment with TPM partially reversed the harmful effects associated with lipid accumulation, even after 3 days pre-treatment with oleate.

The protective effects of TPM on INS-1E β-cells could be explained by enhanced capacity of lipid clearance. Measurements of cellular lipid levels revealed lower fat accumulation in INS-1E cells treated with TPM. The observed enhancement in β-oxidation rates suggests that the mechanism for protection from lipotoxicity could be the inhibition of lipid accumulation in the cytoplasm through increased fatty acid expenditure. Such assumption is strengthened by quantification of the expression of genes involved in lipid and glucose metabolism. Of particular interest, PPARα is a nuclear receptor activated by fatty acid ligands that plays a role as nutrient sensor and transcriptional regulator of lipid metabolism [22,23]. Oleate treatment reduced the expression of PPARα that, remarkably, was elevated upon TPM treatment. PPARα plays a central role in the control of mitochondrial β-oxidation of fatty acids, regulating the transcription of several enzymes such as acyl-CoA oxidase, medium-chain acyl-CoA dehydrogenase and cytochrome P450 [24]. PPARα null mice exhibit reduced capacity to metabolize long-chain fatty acids leading to severe dyslipidemia and large adipose tissue stores [25]. The observed up-regulation of PPARα induced by TPM correlated with augmentation of β-oxidation and is a putative mechanism protecting TPM treated INS-1E β-cells from lipid accumulation and toxicity. In this context, it is of interst that PPARα overexpression in INS-1E cells and rat islets results in potentiation of glucose stimulated insulin secretion [26].

Correlating with the increase in PPARα expression, we observed up-regulation of the fatty acid transporter FAT/CD36. It was recently reported that PPARα could stimulate cellular uptake of fatty acids by increasing the expression of the fatty acid transport protein and fatty acid translocase [27]. Both of these fatty acid transporters might play a role in obesity, diabetes, and in the metabolic syndrome. FAT/CD36 expression is, at least in part, regulated by PPAR isoforms. FAT/CD36 was found to be localized on the mitochondrial membrane of skeletal muscle and heart [28]. Accordingly, it was proposed to be involved in mitochondrial fatty acid oxidation and to be required in the transport of fatty acids across the mitochondrial membrane [29]. FAT/CD36 is also present in the plasma membrane and in the insulin containing granules of β-cells where it could participate to fatty acid uptake and modulation

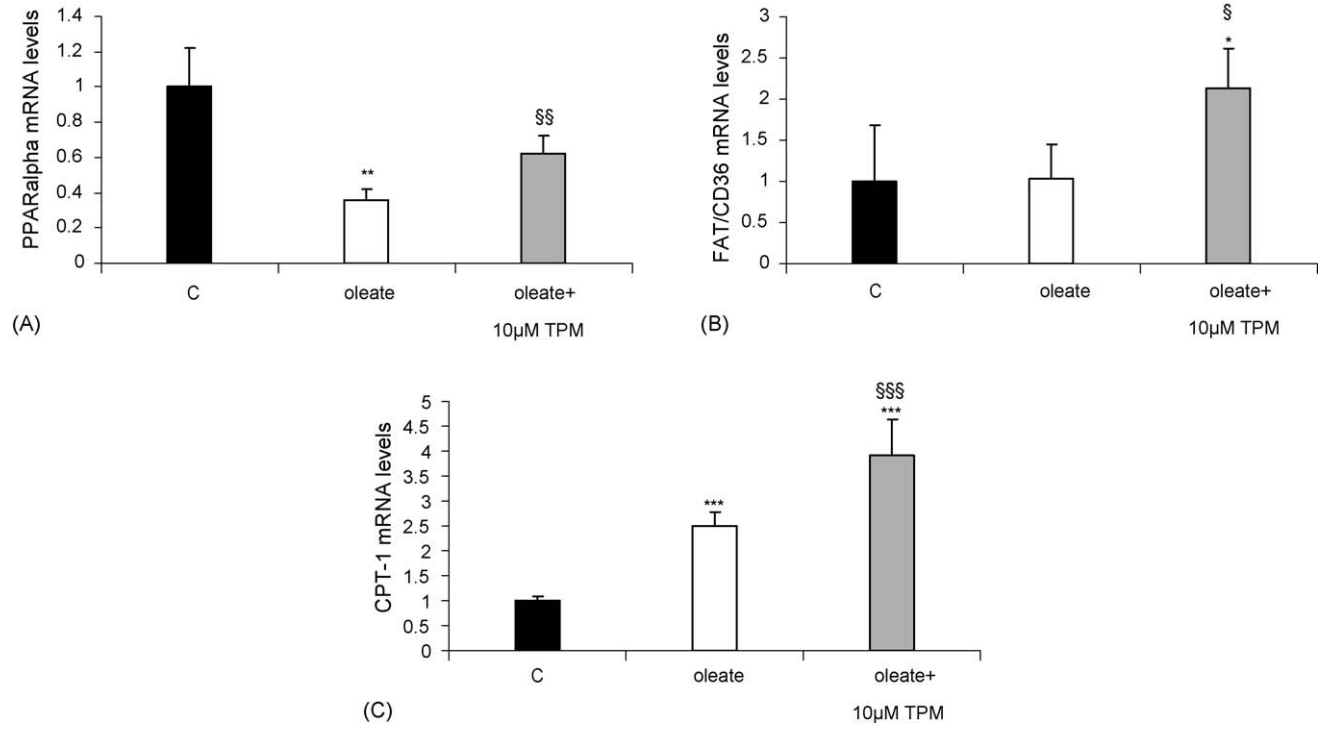

Fig. 7 – Effects of oleate and TPM on gene expression levels in INS-1E cells. Gene expression was measured by quantitative RT-PCR in INS-1E cells treated without (C) or with 0.4 mM oleate (oleate) plus 10 μM TPM (oleate + TPM) for 3 days before total RNA extraction. Expression of genes encoding PPARα (A), FAT/CD36 (B) and CPT-1 (C) were quantified and mRNA levels normalized to those of β-actin. Results are variations from the control presented as means ± S.D. of 4–9 independent experiments. ***,§§§P < 0.01, **,§§P < 0.02, *,§P < 0.05; *vs. control, §vs. oleate).

of insulin secretion [30]. PPARα can increase FAT/CD36 expression by about 80% in the liver, although this effect has not been yet described in β-cells [29]. Once fatty acids are internalized by FAT/CD36, they can be consumed via β-oxidation upon activation of the long chain fatty acid carrier CPT-1 that carries them through the mitochondrial membrane. In our cells, CPT-1 expression was increased by oleate treatment, in accordance with previous reports [21,31]. Interestingly, CPT-1 expression was higher in the presence of TPM, as a possible adaptation to the elevated supply of fatty acids provided by FAT/CD36. CPT-1 over-expression could also be the consequence of PPARα up-regulation, as recently demonstrated in INS-1E β-cells [26].

The impairment of glucose response observed in INS-1E cells exposed to oleate correlated with mitochondrial defects and loss of morphological integrity, both parameters partially reverted by TPM treatment. Several studies proposed a role for mitochondria in lipotoxicity-associated β-cell failure [32]. Indeed, glucose sensing requires preserved oxidative mitochondrial metabolism for the generation of ATP and mitochondrial dysfunction may lead to β-cell failure and type 2 diabetes [33]. Our data are in good agreement with this hypothesis, showing that the recovery of mitochondrial integrity correlated with up-regulation of oxidative genes, such as CPT-1, and was associated with improvement of both mitochondrial membrane potential and glucose stimulated insulin secretion. One of the consequences of lipotoxycity-induced β-cell failure is the induction of apoptosis by fatty acid accumulation [34,35]. In our system, using the monounsaturated fatty acid oleate, there were no signs of apoptotic events (not shown). This is in accordance with the previous observation that β-cell apoptosis is preferentially triggered by saturated fatty acids such as palmitate. Toxicity associated with oleate was more specifically associated with β-cells function rather than cell survival.

In conclusion, we demonstrated that TPM prevented lipotoxicity-induced impairment of glucose stimulated insulin secretion in INS-1E β-cells. This protective effect might be explained by up-regulation of genes involved in β-oxidation and preservation of mitochondrial function. In healthy cells, TPM was without effects and did not modify glucose-stimulated insulin secretion. However, the drug preserved both low insulin release at basal glucose and efficient glucose recognition. This is in accordance with the apparent paradox of weight loss associated with increased insulin levels in glucose tolerance tests. Such new therapeutical approaches might combine treatment of obesity together with protection of β-cell function against lipotoxicity.

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