RESEARCH PAPER

The insulin sensitizing effect of topiramate involves K_ATP channel activation in the central nervous system

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BACKGROUND AND PURPOSE

Topiramate improves insulin sensitivity, in addition to its antiepileptic action. However, the underlying mechanism is unknown. Therefore, the present study was aimed at investigating the mechanism of the insulin-sensitizing effect of topiramate both *in vivo* and *in vitro*.

EXPERIMENTAL APPROACH

Male C57Bl/6J mice were fed a run-in high-fat diet for 6 weeks, before receiving topiramate or vehicle mixed in high-fat diet for an additional 6 weeks. Insulin sensitivity was assessed by hyperinsulinaemic-euglycaemic clamp. The extent to which the insulin sensitizing effects of topiramate were mediated through the CNS were determined by concomitant i.c.v. infusion of vehicle or tolbutamide, an inhibitor of ATP-sensitive potassium channels in neurons. The direct effects of topiramate on insulin signalling and glucose uptake were assessed *in vivo* and in cultured muscle cells.

KEY RESULTS

In hyperinsulinaemic-euglycaemic clamp conditions, therapeutic plasma concentrations of topiramate (~4 μg·mL⁻¹) improved insulin sensitivity (glucose infusion rate + 58%). Using 2-deoxy-D-[³H]glucose, we established that topiramate improved the insulin-mediated glucose uptake by heart (+92%), muscle (+116%) and adipose tissue (+586%). Upon i.c.v. tolbutamide, the insulin-sensitizing effect of topiramate was completely abrogated. Topiramate did not directly affect glucose uptake or insulin signalling neither *in vivo* nor in cultured muscle cells.

CONCLUSION AND IMPLICATIONS

In conclusion, topiramate stimulates insulin-mediated glucose uptake *in vivo* through the CNS. These observations illustrate the possibility of pharmacological modulation of peripheral insulin resistance through a target in the CNS.

Abbreviations

aCSF, artificial cerebrospinal fluid; EGP, endogenous glucose production; GIR, glucose infusion rate; K$_{ATP}$ channel, ATP-sensitive potassium channel; Rd, rate of disposal; T2DM, type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a syndrome characterized by impaired insulin secretion as well as reduced insulin sensitivity. Many drugs have been developed that act on these pathophysiological mechanisms. In recent years, evidence has accumulated that the CNS is also involved in the pathophysiology of T2DM. Experimental models have indicated that insulin-mediated effects on different organs are mediated in part through the CNS (Obici *et al.*, 2002; Koch *et al.*, 2008; Coomans *et al.*, 2011a,b). Importantly, in insulin-resistant conditions, these effects of circulating insulin through the CNS are lost (Coomans *et al.*, 2011a,b). The question arises whether the loss of these effects of insulin, mediated by the CNS, are amendable to pharmacological intervention.

Topiramate, a sulfamate-substituted derivative of the monosaccharide D-fructose (Shank *et al.*, 2000), is used as an antiepileptic drug (McIntyre *et al.*, 2005; Ferrari *et al.*, 2011). The antiepileptic effects of topiramate are mediated by at least six mechanisms of action within the CNS (Zona *et al.*, 1997; Dodgson *et al.*, 2000; Gibbs, III *et al.* 2000; White *et al.*, 1997; 2000; Zhang *et al.*, 2000; Herrero *et al.*, 2002). Studies in obese, diabetic rats demonstrated that topiramate treatment reduced plasma glucose levels and improved insulin sensitivity independently of weight loss (Picard *et al.*, 2000; Wilkes *et al.*, 2005a). However, the mechanism underlying this pharmacological, insulin-sensitizing effect of topiramate is unknown.

We hypothesized that topiramate improves insulin sensitivity not by a direct effect on peripheral organs, but, rather, through effects within the CNS. Therefore, we studied in high-fat fed mice the effects of i.c.v. administered vehicle versus tolbutamide on top of the effects of topiramate on tissue-specific insulin-mediated glucose uptake. Tolbutamide is an inhibitor of ATP-sensitive potassium (K$_{ATP}$) channels in neurons and i.c.v. administration of tolbutamide blocks the action of circulating insulin in the brain (Obici *et al.*, 2002; Coomans *et al.*, 2011a,b). In addition, we assessed the direct effects of topiramate on insulin signalling and glucose uptake *in vivo* and in cultured C2C12 muscle cells. In this study, we show that topiramate improves peripheral insulin sensitivity to a large extent by improving insulin sensitivity in the brain.

Methods

Animals

Eighty-six male C57Bl/6J mice obtained from Charles River Laboratories at an age of 8 weeks were housed in a temperature-controlled room on a 12 h light-dark cycle. From the age of 12 weeks, mice were fed *ad libitum* a run-in high-fat diet for 6 weeks (45 energy% of fat derived from lard; Research Diets Inc, New Brunswick, NJ, USA), which induces considerable insulin resistance, according to our previous experiments (van den Berg *et al.*, 2010). Subsequently, the animals were randomized according to body weight and fasting plasma glucose levels and were fed *ad libitum* for 6 weeks a high-fat diet containing 3.33% anise (anise cubes, De Ruijter, Utrecht, The Netherlands) with (*n* = 42) or without (*n* = 44) 0.12% (w/w) topiramate (Abbott Products GmbH, Hannover, Germany). The mice had free access to water throughout the experiment. Food intake and body weight were measured regularly throughout the experiment. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center, Leiden, The Netherlands approved the protocol. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

I.c.v. cannula implantation

For i.c.v. cannula implantation, 15-week-old male mice were anaesthetized with 0.5 mg·kg^{-1} Medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg·kg^{-1} Midazolam (Roche, Mijdrecht, The Netherlands) and 0.05 mg·kg^{-1} Fentanyl (Janssen-Cilag, Tilburg, The Netherlands) and placed in a stereotactic device (TSE systems, Homburg, Germany). A 25 gauge guide cannula was implanted into the left lateral ventricle using the following coordinates from Bregma: 1.0 mm lateral, 0.46 mm posterior and 2.2 mm ventral. The guide cannula was secured to the skull surface with dental cement (GC Europe N.V., Leuven, Belgium). Analgesia (0.03mg·kg^{-1} Buprenorphine (Schering-Plough, Houten, The Netherlands) was administered and anesthesia was antagonized using 2.5 mg·kg^{-1} Antipamezol (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 mg·kg^{-1} Flumazenil (Roche, Mijdrecht, The Netherlands) and 1.2 mg·kg^{-1} Naloxon (Orpha, Purkersdorf, Austria). After a recovery period of 1 week, cannula placement was verified. Mice that ate >0.3 g in 1 h in response to i.c.v. injection of 5 μg NPY (Bachem, St. Helens, UK) in 1 μl of artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Natick, MA, USA) were considered to have the cannula correctly placed and were included in the study (van den Hoek *et al.*, 2004; Coomans *et al.*, 2011b).

Hyperinsulinaemic-euglycaemic clamp studies

Overnight fasted, body weight-matched male mice (vehicle-treated mice *n* = 10; topiramate-treated mice *n* = 7) were anaesthetized with 6.25 mg·kg^{-1} Acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg·kg^{-1} Midazolam (Roche), and 0.31 mg·kg^{-1} Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Anaesthesia, as well as body temperature, was maintained throughout the procedure. First, basal rates of glucose turnover were determined by administration of a primed continuous i.v. infusion of D-[1-^{14}C]glucose (0.3 μCi·kg^{-1}·min^{-1}; Amersham, Little Chalfont, UK) for 60 min. Subsequently, insulin (Actrapid, Novo Nordisk, Denmark) was administered i.v. by primed (4.1 mU), continuous (6.8 mU·h^{-1}) infusion to attain steady-state hyperinsulinemia

together with D-[1-^{14}C]glucose (0.3 µCi·kg^{-1}·min^{-1}; Amersham) for 90 min. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10-min intervals via tail bleeding (<3 µL, Accu-chek, Sensor Comfort; Roche Diagnostics, Mannheim, Germany). To assess insulin-mediated glucose uptake in individual tissues, 2-deoxy-D-[3-^{3}H]glucose (2-[^{3}H]DG; Amersham) was administered as a bolus (1 µCi) 30 min before the end of both experiments. In the last 20 min of both experiments, blood samples were taken with intervals of 10 min. Subsequently, the mice were sacrificed by cervical dislocation and after perfusion with ice-cold PBS, organs were harvested and snap-frozen in liquid nitrogen.

I.c.v. tolbutamide treatment during clamp

Starting 30 min before the initiation of the hyperinsulinaemic-euglycaemic clamp, aCSF (vehicle-treated mice n = 8; topiramate-treated mice n = 10) or the K$_{ATP}$ channel blocker tolbutamide (vehicle-treated mice n = 7; topiramate-treated mice n = 9), dissolved in 5% DMSO to a final concentration of 4.8 mM in aCSF, was continuously infused i.c.v. at a rate of 2.5 µL per hour (Obici et al., 2002; Plum et al., 2006). The concentration of i.c.v. tolbutamide was ascertained in a dose-finding study, to ensure that tolbutamide did not leak to the periphery.

In vivo insulin signalling in muscle

Overnight fasted mice were anaesthetized and received either an i.v. bolus of insulin (4.1 mU; vehicle-treated mice n = 10; topiramate-treated mice n = 8) or saline (vehicle-treated mice n = 9; topiramate-treated mice n = 8). After 10 min, the mice were sacrificed by cervical dislocation and skeletal muscles (upper limb) were harvested and snap-frozen in liquid nitrogen. Skeletal muscles were homogenized by Ultra-Turrax (22.000 rpm; 2 × 5 s) in a 10:1 (v/w) ratio of ice-cold buffer containing: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.6), 50 mM sodium fluoride, 50 mM potassium chloride (KCl), 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 5 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol, 1% nonyl phenoxypoly-ethoxylethanol (Tergitol-type NP40) and protease inhibitors cocktail (Complete, Roche, Mijdrecht, The Netherlands). The effect of topiramate on the canonical insulin signalling pathway was subsequently determined by Western blot analysis.

Plasma analysis

Blood samples were taken from the tail tip into chilled paraoxon-coated capillaries to prevent ex vivo lipolysis. The tubes were immediately placed on ice and centrifuged at 4°C. Plasma levels of glucose and free fatty acids (FFA) were determined using commercially available kits and standards according to the instructions of the manufacturer (Instruchemie, Delfzijl, The Netherlands). Plasma insulin levels were measured using a mouse-specific insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). Total plasma ^{14}C-glucose and ^{3}H-glucose were determined in supernatant of 7.5 µL plasma, after protein precipitation using 20% trichloro-acetic acid and evaporation to eliminate tritiated water.

Tissue analysis

For determination of tissue 2-[^{3}H]DG uptake, homogenates of heart, skeletal muscle and adipose tissue were boiled, and the supernatants were subjected to an ion-exchange column [described previously (Rossetti et al. 1990; van den Hoek et al., 2008; Parlevliet et al., 2009)] to isolate 2-[^{3}H]DG-6-phosphate, a metabolic end-product of 2-[^{3}H]DG that accumulates in muscle and fat cells.

C2C12 cells

C2C12 skeletal muscle cells were cultured in a humidified atmosphere containing 5% CO$_2$ in DMEM containing 25 mM glucose, glutamine and pyruvate (Invitrogen, Bleiswijk, The Netherlands) supplemented with 10% FBS and 100 units per mL penicillin and 100 µg·mL^{-1} streptomycin (Invitrogen). For the deoxyglucose uptake assay ~15 000 cells per well were seeded and cultured on 12-well plates. For Western blot analysis ~25 000 cells per well were seeded and cultured on 6-well plates. After reaching confluence, the cells were differentiated into myotubes by replacing the complete growth medium with differentiation medium (same DMEM medium containing antibiotics, but supplemented with 2% horse serum instead of 10% FBS). The differentiation medium was changed every 48 h. The myotubes were used for experiments at day 7 of differentiation.

Deoxyglucose uptake assay in C2C12 myotubes

C2C12 myotubes were serum-starved for 4 h before the experiment. After serum starvation, cells were washed once with PBS and once with buffer (50 mM HEPES, 138 mM sodium chloride, 1.85 mM calcium chloride, 1.3 mM magnesium sulfate and 4.8 mM KCl, pH 7.4) followed by incubation with the same buffer for 45 min at 37°C in the presence of topiramate (1 or 100 µM) or vehicle. At the end of incubation, cells were challenged with or without 1 µM insulin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min in the presence of topiramate or vehicle. 2-Deoxy-D-[1-^{14}C] glucose (2DG) was added to the cells for another 10 min (0.012 µCi per dish). The reaction was ended by washing three times with ice-cold PBS and addition of a lysis buffer containing 1% sodium dodecyl sulfate (SDS)/0.2 M sodium hydroxide. The lysates were transferred into plastic vials, 2 mL of scintillation liquid (Instagel Plus, PerkinElmer, Waltham, MA, USA) was added and radioactivity was measured in a scintillation counter.

Insulin signalling in C2C12 myotubes

C2C12 myotubes were serum-starved for 4 h then washed once with PBS and once with 2DG buffer followed by incubation with the same buffer for 45 min at 37°C in the presence of topiramate (1 or 100 µM) or vehicle. At the end of incubation, cells were stimulated with 1 µM insulin. After 20 min, cells were rapidly washed one time with ice-cold PBS and lysed by addition of a buffer containing 12.5% glycerol, 3% SDS and 100 mM TrisPO$_4$, pH 6.8. The cell lysates were then immediately boiled for 5 min and stored at –20°C until use.

Palmitate-induced insulin resistance in C2C12 myotubes

Palmitate-containing medium was prepared to induce insulin resistance in C2C12 myotubes. DMEM basic medium was

supplemented with antibiotics and 2% FFA-free BSA. Palmitate was first dissolved in ethanol and then added to medium containing BSA at a final concentration of 0.75 mM. The final medium was sonicated for 5 min and warmed at 55°C for 10 min to allow complex formation between BSA and palmitate. Differentiated myotubes (day 6) were then treated with palmitate for 16 h before performing deoxyglucose uptake assay.

Western blot analysis

Protein content in cell or tissue homogenates was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Proteins (10 μg) were separated by 10% SDS-PAGE followed by transfer to a PVDF transfer membrane. Membranes were blocked for 1 h at room temperature in Tris Buffer Saline Tween20 (TBST) buffer with 5% non-fat dry milk followed by an overnight incubation with antibodies (all from Cell Signaling Technology, Beverly, MA, USA). Blots were then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence and quantified using Image J (NIH, US).

Calculations

Turnover rates of glucose ($\mu mol \cdot min^{-1} \cdot kg^{-1}$) were calculated in basal and hyperinsulinaemic conditions as the rate of tracer infusion ($dpm \cdot min^{-1}$) divided by plasma specific activity of ^{14}C-glucose ($dpm \cdot \mu mol^{-1}$). The ratio was corrected for body weight. Endogenous glucose production was identical to the glucose appearance rate under basal conditions and calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate under hyperinsulinaemic-euglycaemic clamp conditions.

Tissue-specific glucose uptake in heart, skeletal muscle and adipose tissue was calculated from tissue 2-[^3H]DG content, corrected for plasma specific activity and expressed as micromoles per gram of tissue.

Statistical analysis

Differences between groups were determined by Mann–Whitney non-parametric tests for two independent samples. The criterion for significance was set at $P < 0.05$. All values shown represent means ± SEM.

Results

In vivo studies

After 6 weeks of run-in high-fat diet, mice were randomized on body weight (25.5 ± 0.4 vs. 26.1 ± 0.8 g) and fasting plasma glucose levels (4.2 ± 0.2 vs. 4.1 ± 0.2 $mmol \cdot L^{-1}$) and received high-fat diet containing topiramate or vehicle during 6 subsequent weeks. After 5 weeks of topiramate treatment, the topiramate concentration in plasma, determined by liquid chromatography tandem mass spectrometry assay as previously described (Christensen et al., 2002), was 3.7 ± 0.2 $\mu g \cdot mL^{-1}$ (n = 7). After 6 weeks of topiramate treatment, the topiramate concentration in the brains of overnight fasted mice was 115 ± 18 ng per g brain tissue (n = 23).

Table 1

Results obtained during the hyperinsulinaemic-euglycaemic clamp study in vehicle- (n = 10) and topiramate-treated (n = 7) animals. Data are represented as means ± SEM. There were no significant differences between vehicle- and topiramate-treated animals. *$P <$ 0.05 basal versus hyperinsulinaemic-euglycaemic clamp period

	Vehicle	Topiramate
Body weight (g)	25.8 ± 0.4	24.9 ± 0.8
Basal haematocrit (%)	40.3 ± 0.2	38.5 ± 0.2
Clamp haematocrit (%)	36.8 ± 0.2	34.9 ± 0.5
Basal insulin ($ng \cdot mL^{-1}$)	0.5 ± 0.1	0.5 ± 0.1
Clamp insulin ($ng \cdot mL^{-1}$)	5.1 ± 0.4*	4.3 ± 0.5*
Basal glucose ($mmol \cdot L^{-1}$)	4.1 ± 0.1	4.2 ± 0.1
Clamp glucose ($mmol \cdot L^{-1}$)	4.5 ± 0.3	4.0 ± 0.2

Effect of topiramate on insulin sensitivity in mice. After 6 weeks of topiramate treatment, insulin sensitivity was assessed using hyperinsulinaemic-euglycaemic clamp studies. Body weight did not differ between topiramate- (n = 7) and vehicle-treated (n = 10) mice (Table 1). The reduction in body weight was also not statistically different between topiramate- (−2.9%) and vehicle-treated mice (−4.7%). In the basal period of the hyperinsulinaemic-euglycaemic clamp, endogenous glucose production (EGP), which equals glucose disposal (Rd), was not different between topiramate- and vehicle-treated animals.

The specific activity of ^{14}C-glucose measured at 10-min intervals indicated the presence of steady-state conditions in both groups (Supporting Information Table S1). The glucose infusion rate (GIR) necessary to maintain euglycemia was 50% higher in topiramate-treated animals compared to vehicle-treated animals (average GIR 84 ± 8 vs. 56 ± 4 $\mu mol \cdot min^{-1} \cdot kg^{-1}$ for the last 20 min of the experiment, $P <$ 0.05, Figure 1A), in the presence of similar glucose levels (Table 1, Supporting Information Fig. S1), indicating that topiramate improved insulin sensitivity.

In the hyperinsulinaemic-euglycaemic period, insulin inhibited EGP to the same extent in both groups of mice (92 ± 8 vs. 80 ± 7%, n.s., Figure 1B). Insulin-mediated Rd, however, was increased by 104% in the topiramate-treated group compared to the vehicle-treated group (96 ± 16 vs. 47 ± 9%, respectively, $P <$ 0.05, Figure 1C), indicating that topiramate treatment improved peripheral insulin sensitivity.

Effect of i.c.v. administration of tolbutamide on the effects of topiramate on insulin sensitivity in mice. To determine whether topiramate improved peripheral insulin sensitivity by affecting insulin signalling in the brain, tolbutamide (vehicle-treated mice n = 7; topiramate-treated mice n = 9), an inhibitor of K_{ATP} channels in neurons, or vehicle (vehicle-treated mice n = 8; topiramate-treated mice n = 10) was infused into the lateral ventricle (i.c.v.) during hyperinsulinaemic-euglycaemic clamp experiments. The specific activity in plasma of ^{14}C-glucose measured at 10-min intervals indicated the presence of steady-state conditions in

A GIR (Av last 20 min)

B Inhibition glucose production

C Stimulation glucose uptake

Figure 1
Glucose infusion rate (GIR, A), endogenous glucose production (EGP, B) and glucose disposal (Rd, C) in vehicle-treated (white) and topiramate-treated (black) animals as measured in hyperinsulinaemic-euglycaemic clamp studies. Values represent means ± SEM for $n = 7$–10. *$P < 0.05$ topiramate versus vehicle. Av, average.

all groups (Supporting Information Table S2). In agreement with the first experiment, topiramate-treated animals receiving vehicle (aCSF) had higher GIR compared to vehicle-treated animals receiving aCSF (average GIR 85 ± 9 vs. 55 ± 11 μmol·min^{-1}·kg^{-1} for the last 20 min of the experiment, $P < 0.05$, Figure 2A), in the presence of similar glucose levels (Table 2, Supporting Information Figure S2). I.c.v. tolbutamide administration in vehicle-treated animals did not affect any of the parameters tested compared to i.c.v. aCSF infused vehicle-treated animals (Figure 2A–D), in line with our previous study in high-fat fed mice (Coomans et al., 2011a). In topiramate-treated animals, i.c.v. administration of tolbuta-

mide decreased GIR compared to i.c.v. aCSF (average GIR 75 ± 7 vs. 85 ± 9 μmol·min^{-1}·kg^{-1} for the last 20 min of the experiment, $P < 0.05$, Figure 2A), indicating that i.c.v. tolbutamide counteracted, at least in part, the improvement in insulin sensitivity induced by topiramate. In the basal period as well as in the hyperinsulinaemic period, EGP was not different between all groups of mice (Figure 2B). Insulin-stimulated Rd was again significantly higher in topiramate-treated animals compared to vehicle-treated animals receiving aCSF (101 ± 15 vs. 71 ± 12%, respectively, $P < 0.05$, Figure 2C). However, i.c.v. administration of tolbutamide in topiramate animals diminished the improvement of Rd induced by the drug (81 ± 12 vs. 101 ± 15%, respectively, $P < 0.05$, Figure 2C). Assessment of tissue-specific 2-[^{3}H]DG uptake revealed that topiramate treatment increased insulin-stimulated glucose uptake in heart, skeletal muscle and adipose tissue (gonadal fat pad) (Figure 2D). Interestingly, i.c.v. tolbutamide in topiramate-treated animals abrogated the improvement in insulin-stimulated 2-[^{3}H]DG uptake in heart, skeletal muscle and adipose tissue.

Effect of topiramate on muscle insulin signalling in mice. To determine the direct effect of topiramate on muscle, we determined insulin signalling in skeletal muscle (upper hindlimb) 10 min after insulin (vehicle-treated mice $n = 10$; topiramate-treated mice $n = 8$) or saline i.v. injection (vehicle-treated mice $n = 9$; topiramate-treated mice $n = 8$). As expected, insulin strongly increased the phosphorylation state of PKB and proline-rich Akt substrate of 40 kDa in muscle (Figure 3A-C). The expression of insulin receptor β (IRβ) and its downstream signalling pathway were not differently affected between vehicle and topiramate-treated mice. Insulin decreased the phosphorylation state of AMP-activated protein kinase (AMPK) in vehicle-treated mice as expected (Figure 3D). In topiramate-treated mice, AMPK phosphorylation was increased under insulin-stimulated conditions compared to vehicle-treated mice.

In vitro *studies*

Effect of topiramate on glucose uptake in differentiated myotubes. To exclude that topiramate directly increased glucose uptake at the tissue level, we investigated the direct effects of topiramate on glucose uptake in differentiated C2C12 myotubes (Figure 4A). Cells were treated with increasing concentrations of topiramate (1 or 100 μM) or vehicle for 45 min and glucose uptake was then measured during the last 20 min, after addition, or not, of 1 μM insulin. Insulin stimulated glucose uptake by about +25% in control condition, that is without topiramate. Topiramate did not increase basal or insulin-stimulated glucose uptake (Figure 4A).

Effect of topiramate on insulin signalling in differentiated myotubes. Western blot analyses were performed to determine whether topiramate affects the insulin signalling pathway in differentiated myotubes by assessing the phosphorylation state of PKB (Figure 4B). As expected, insulin strongly increased the phosphorylation state of PKB on Ser^{473} (+583%) in control condition. Topiramate did not affect basal or insulin-stimulated phosphorylation of PKB. (Figure 4B).
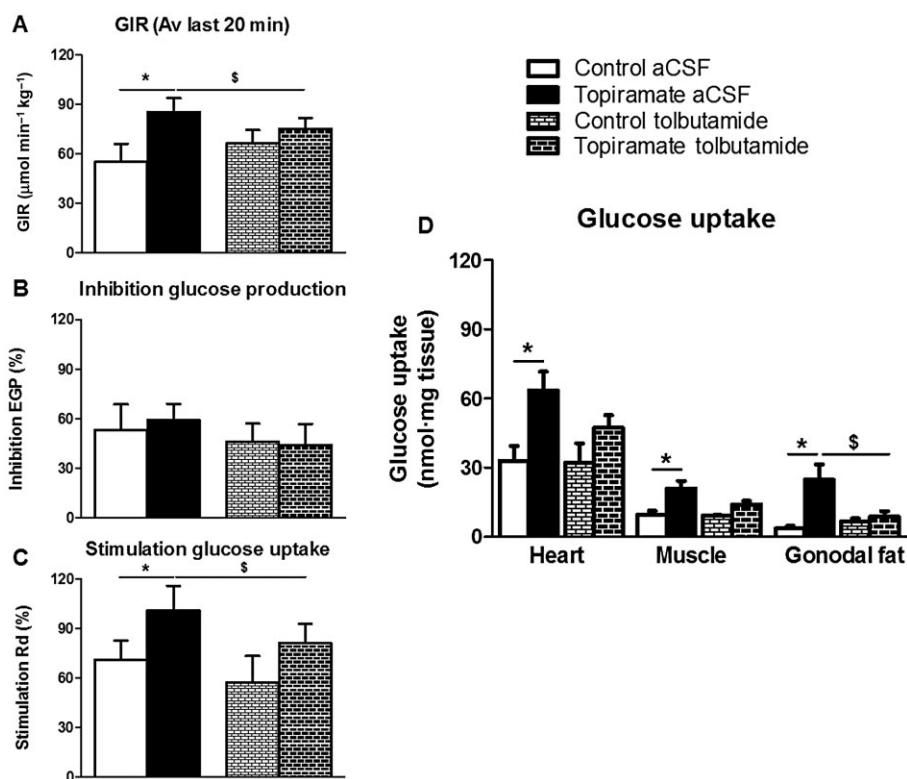
Figure 2

Glucose infusion rate (GIR, A), endogenous glucose production (EGP, B), glucose disposal (Rd, C) and organ-specific glucose uptake (D) in vehicle-treated (white) and topiramate-treated animals (black) receiving i.c.v. vehicle (artificial cerebrospinal fluid, aCSF) or tolbutamide. Values represent means ± SEM for n = 7–10. *P < 0.05 topiramate versus vehicle. $^$$P$ < 0.05 i.c.v. tolbutamide versus vehicle. Av, average.
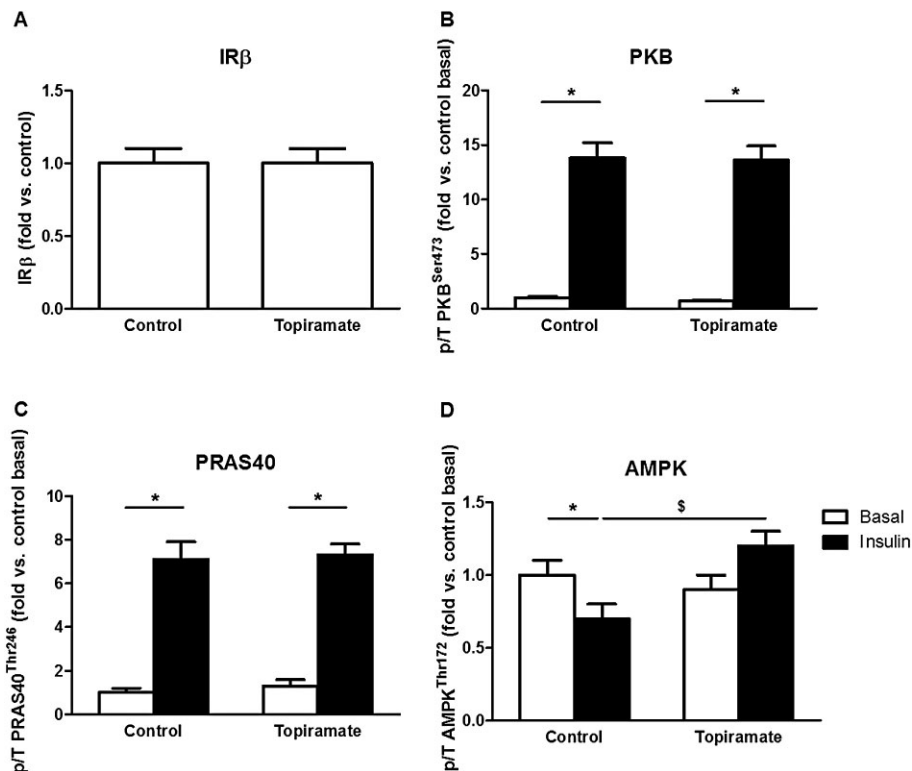
Table 2

Results of i.c.v. administration of aCSF (vehicle-treated mice n = 8; topiramate-treated mice n = 10) and tolbutamide (vehicle-treated mice n = 7; topiramate-treated mice n = 9) obtained from the hyperinsulinaemic-euglycaemic clamp study in vehicle- and topiramate-treated animals. Data are represented as means ± SEM. There were no significant differences between vehicle- and topiramate-treated animals. *P < 0.05 basal versus hyperinsulinaemic-euglycaemic clamp period. aCSF, artificial cerebrospinal fluid; FFA, free fatty acids

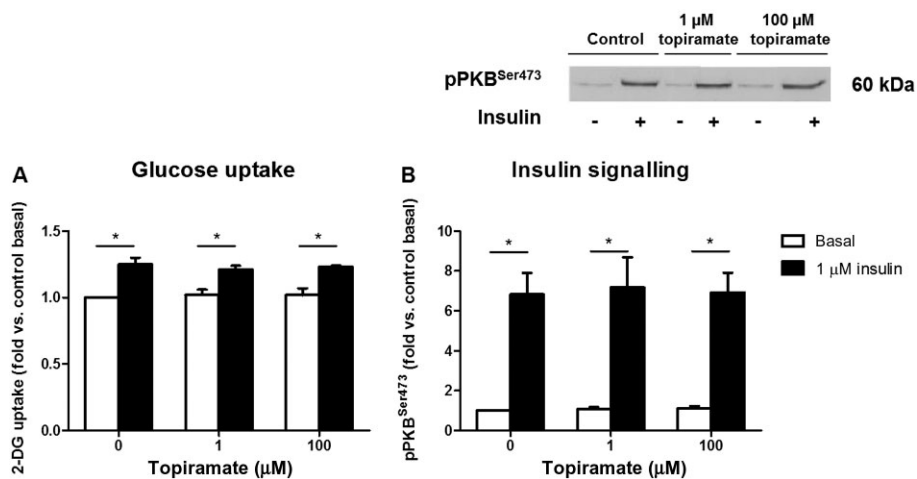|	Vehicle		Topiramate	
	aCSF	**Tolbutamide**	**aCSF**	**Tolbutamide**
Body weight (g)	28.6 ± 0.7	28.7 ± 0.7	27.7 ± 0.5	27.8 ± 0.3
Basal haematocrit (%)	40.3 ± 0.2	39.0 ± 0.8	38.5 ± 0.2	37.1 ± 0.3
Clamp haematocrit (%)	36.8 ± 0.2	36.3 ± 0.2	34.9 ± 0.5	34.4 ± 0.4
Basal insulin (ng·mL^{-1})	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Clamp insulin (ng·mL^{-1})	4.1 ± 0.4*	3.3 ± 0.1*	3.4 ± 0.2*	3.8 ± 0.4*
Basal glucose (mmol·L^{-1})	5.0 ± 0.2	5.4 ± 0.3	5.5 ± 0.2	4.9 ± 0.2
Clamp glucose (mmol·L^{-1})	5.6 ± 0.3	6.0 ± 0.5	5.8 ± 0.3	5.2 ± 0.3
Basal FFA (mmol·L^{-1})	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0
Clamp FFA (mmol·L^{-1})	0.3 ± 0.0*	0.3 ± 0.0*	0.3 ± 0.0*	0.3 ± 0.0*

Effect of topiramate on glucose uptake in insulin-resistant myotubes. Next, we investigated whether topiramate was able to reverse palmitate-induced insulin resistance in C2C12 myotubes (Figure 5). After incubation with palmitate for 16 h,

C2C12 myotubes were incubated with increasing concentrations of topiramate (1 or 100 μM) for 45 min and challenged with or without 1 μM of insulin for 20 min. At maximal concentration of insulin, glucose uptake was increased by

Figure 3

Expression of IRβ (A) and phosphorylation state of PKB (B), PRAS40 (C) and AMPK (D) in basal state (black bars) or hyperinsulinaemic state (white bars) in muscle of mice-treated with vehicle or topiramate. The Western blot data were expressed as fold change compared to vehicle at basal condition. Data are represented as means ± SEM for n = 8–10, *P < 0.05 insulin versus basal. $^{\$}$$P$ < 0.05 topiramate versus vehicle.
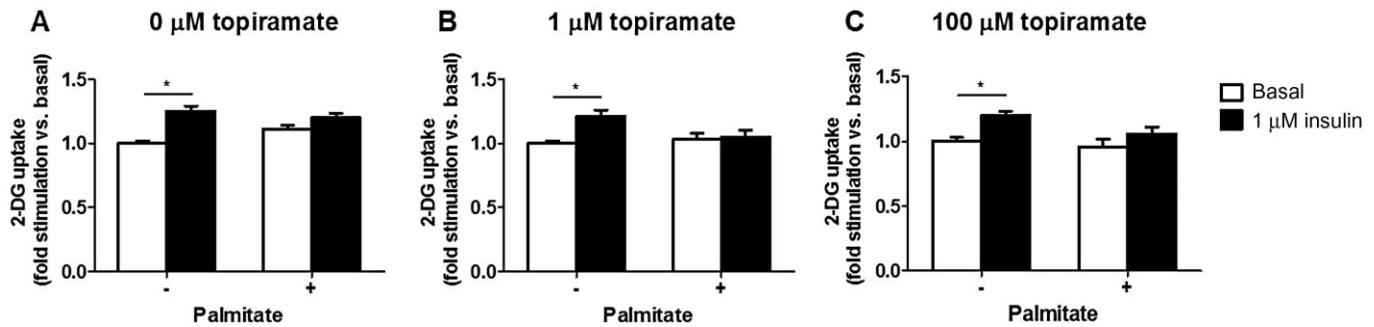

Figure 4

Glucose uptake (A) and insulin signalling (B,C) in C2C12 myotubes at basal (white) or insulin-stimulated (black) condition with topiramate (1 or 100 μM) or vehicle. The quantification was normalized for total protein and expressed as fold change compared to vehicle at basal condition. Data are represented as means ± SEM for n = 3–4, *P < 0.05 insulin versus basal.

+25% in control condition, that is without palmitate and topiramate (Figure 4A). Preincubation with palmitate resulted in a decrease of insulin-stimulated glucose uptake. There was no effect of topiramate on basal or insulin-stimulated glucose uptake (Figure 5B,C).

Discussion and conclusions

In the present study, topiramate improved insulin sensitivity by increasing glucose uptake by skeletal and cardiac muscle and by adipose tissue in high-fat fed mice. In addition,

Figure 5

Glucose uptake (A-C) by healthy or palmitate-induced insulin resistant C2C12 myotubes at basal (white) or insulin-stimulated (black) condition with topiramate (1 or 100 µM) or vehicle. The quantification was normalized for total protein and expressed as fold change compared to vehicle at basal condition. Data are represented as means ± SEM for $n = 3–4$, $*P < 0.05$ insulin versus basal.

inhibition of the central action of circulating insulin by i.c.v. administration of tolbutamide, a K_{ATP} channel blocker in neurons, prevented this insulin-sensitizing effect of topiramate. Topiramate had no direct effect on muscle insulin signalling and glucose uptake *in vivo* and *in vitro*. Collectively, these data indicate that topiramate improves peripheral insulin sensitivity indirectly via the brain, rather than by directly targeting peripheral organs.

The mice were fed a high-fat diet for 6 weeks to reduce insulin sensitivity before topiramate treatment was started, as topiramate exerts its greatest effects in obese, insulin resistant subjects (Astrup and Toubro, 2004). The half-life of topiramate in humans is 21 h, whereas the half-life in rodents is only 1–2 h (Bialer *et al.*, 2004). To obtain stable concentrations of topiramate in plasma of our mice, topiramate was mixed through the diet with addition of 3.33% anise to cover the bitter taste. The dose of topiramate used in the present study resulted in plasma concentrations within the therapeutic range (~4 µg·mL^{-1}). Hyperinsulinaemic-euglycaemic clamp analyses revealed that topiramate improved insulin sensitivity independent of weight loss, in agreement with previous studies (Wilkes *et al.*, 2005a,b; Stenlof *et al.*, 2007). In the present study, we extend these observations by showing that this insulin-sensitizing effect of topiramate was present in cardiac and skeletal muscle, as well as in adipose tissue. In contrast, topiramate did not improve hepatic insulin sensitivity. This improved insulin sensitivity in muscle and adipose tissue, but not in liver, has previously been associated with enhanced AMPK phosphorylation in muscle, but not in liver (Wilkes *et al.*, 2005b). Also in the current study, we show enhanced AMPK phosphorylation in skeletal muscle upon topiramate treatment. As α-adrenergic stimulation enhances AMPK phosphorylation, the increased glucose uptake by peripheral organs might be related to increased sympathetic nervous system activation (Minokoshi *et al.*, 2002). The topiramate concentration in plasma correlates with that in CSF (Christensen *et al.*, 2001). Moreover, the effects of topiramate on body weight, body composition and energy metabolism have been associated with altered neuropeptide expression in the hypothalamus (York *et al.*, 2000). Combined with the absence of direct effects of topira-

mate on insulin sensitivity in muscle cells, we therefore hypothesized that the brain mediated the effects of topiramate on insulin sensitivity.

Topiramate has been shown to act at the level of the central nervous system by modifying and inhibiting neurotransmission: (i) enhancement of GABA-ergic activity (White *et al.*, 1997; 2000), (ii) inhibition of kainite/AMPA receptors (Gibbs *et al.*, 2000), (iii) inhibition of voltage-dependent sodium channels (Zona *et al.*, 1997), (iv) inhibition of high-voltage-activated calcium channels (Zhang *et al.*, 2000) and (v) inhibition of carbonic anhydrase (Dodgson *et al.*, 2000). As a result of carbonic anhydrase inhibition, potassium currents are activated (Herrero *et al.*, 2002). Potassium (K+) channels are membrane-spanning proteins that selectively conduct K+ ions across the cell membrane. There are several classes of K+ channels, including the voltage-gated potassium channels and the inwardly rectifying potassium channels. Although topiramate has effects on voltage-gated potassium channels (Danielsson, *et al.*, 2005), topiramate has no direct effect on the inwardly rectifying G-protein coupled or the K_{ATP} channels (Kobayashi *et al.*, 2009).

K_{ATP} channels are widely expressed in the cytoplasmic membranes of neurons and play an important role in the regulation of cell excitability (Ashcroft and Gribble, 1998). These channels open when the intracellular ATP to ADP ratio decreases, resulting in membrane hyperpolarization and close when this ratio increases, resulting in membrane depolarization. The K_{ATP} channel is composed of the sulfonylurea receptor type-1 (SUR1) and the inwardly rectifying K+ channel (Kir6.2) subunits (Seino and Miki, 2003). Tolbutamide binds to the SUR1 subunit of the K_{ATP} channel, thereby preventing opening of this channel (Babenko *et al.*, 2000; Moreau *et al.*, 2005). We and others have shown that in lean animals, tolbutamide blocks activation of K_{ATP} channels by insulin (Spanswick *et al.*, 2000; Obici *et al.*, 2002; Coomans *et al.*, 2011b). In obese animals, however, hypothalamic K_{ATP} channels are insensitive to insulin and blockage of the K_{ATP} channels by tolbutamide has no effect (Coomans *et al.*, 2011a,b). To test our hypothesis that the CNS is involved in the anti-diabetic effects of topiramate by improving insulin signalling in the brain, we administered tolbutamide i.c.v.

in topiramate-treated animals on a high-fat diet during hyperinsulinaemic-euglycaemic clamp conditions. In the present study, high-fat feeding resulted in similar central insulin resistance as these previous studies, since i.c.v. tolbutamide in vehicle-treated animals had no effect on insulin-inhibited glucose production or insulin-stimulated glucose uptake. Interestingly, we show that topiramate is able to restore hypothalamic insulin sensitivity, thereby restoring hypothalamic sensitivity to K_{ATP} channels blockage by tolbutamide. This is in line with a recent study showing that short-term topiramate treatment improved hypothalamic insulin signalling (Caricilli et al., 2012). As topiramate has no direct effects on K_{ATP} channels (Kobayashi et al., 2009), the exact mechanism by which topiramate restores hypothalamic insulin sensitivity remains to be identified.

Peripheral and hypothalamic insulin effects are both required for physiological regulation of glucose homeostasis. Activation of K_{ATP} channels in hypothalamic neurons by insulin provides a signal inhibiting gluconeogenesis, that is conveyed to the liver by parasympathetic vagal fibres. K_{ATP} channels, however, are not only expressed in neurons of different brain regions, but also in microglial cells (McLarnon et al., 2001). In activated microglia, the K_{ATP} channel components SUR-1 and SUR-2 are present together with glucokinase, suggesting that microglia also have gluco-sensing properties (Ramonet et al., 2004). This energy-sensing capacity might be a key element for controlling microglial activation and secretion of proinflammatory cytokines following CNS injury. However, until now, the function of these K_{ATP} channels in microglial cells has not been studied in relation to peripheral glucose homeostasis. We cannot exclude the possibility that such mechanisms in microglial cells might be involved in the results presented in the current study.

Topiramate treatment did not exert any direct effects on insulin signalling in muscle in vivo. Furthermore, both basal or insulin-stimulated glucose uptake and insulin signalling in insulin-sensitive or insulin-resistant cultured muscle cells (differentiated C2C12 myotubes) were not affected by topiramate. Our data are in contrast to a previous study that reported that topiramate increases glucose uptake in cultured insulin-sensitive L6 cells, a rat skeletal muscle cell line, via an AMPK-mediated pathway (Ha et al., 2006). The phosphorylation level of AMPK (Thr172) was not increased when we treated C2C12 myotubes with topiramate (data not shown). The apparent contradiction between the previous study performed in L6 cells and our study could be due to difference in origin of cell-type, the differentiation status of the muscle cells or other differences in experimental conditions.

In conclusion, topiramate improves insulin sensitivity in high-fat fed mice by stimulating insulin-mediated glucose uptake by skeletal muscle, heart and adipose tissue through effects within the CNS. Inhibition of K_{ATP} channel activation in the brain abrogates the insulin-sensitizing effect of topiramate in these mice. These observations indicate that the anti-diabetic effects of topiramate are the result of actions in the brain, rather than of direct effects of topiramate on peripheral organs. These observations demonstrate the possibility of pharmacological treatment of peripheral insulin resistance through targets in the CNS.

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Hyperinsulinaemic-euglycaemic clamp: glucose concentrations (A) and glucose infusion rates (GIR, B). Values represent means ± SEM for 7–10 mice per group. *$P < 0.05$, topiramate versus vehicle.

Figure S2 Hyperinsulinaemic-euglycaemic clamp: glucose concentrations (A) and glucose infusion rates (GIR, B). Values represent means ± SEM for 7–10 mice per group. *$P < 0.05$, topiramate versus other groups.

Table S1 Specific activity of ^{14}C-glucose (dpm·mmol^{-1}) in the basal period or in the hyperinsulinaemic-euglycaemic period. Values are means ± SEM for $n = 7$–10.

Table S2 Specific activity of ^{14}C-glucose (dpm·mmol^{-1}) in the basal period or in the hyperinsulinaemic-euglycaemic period. Values are means ± SEM for $n = 7$–10.