THE INSULIN SENSITIZING EFFECT OF TOPIRAMATE INVOLVES K_{ATP} CHANNEL ACTIVATION IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT

Topiramate is associated with improvement in insulin sensitivity, in addition to its antiepileptic action. However, the mechanism underlying this insulin-sensitizing effect of topiramate is unknown. In the present study, we investigated the insulin-sensitizing effects of topiramate and the underlying mechanism both *in vivo* and *in vitro*.

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 6 weeks. In basal and hyperinsulinemic-euglycemic clamp conditions, whole body glucose kinetics was measured using D-[^{14}C]glucose and tissue-specific glucose uptake using 2-deoxy-D-[^{3}H]glucose. In addition, we determined the extent to which these effects of topiramate were mediated through the central nervous system by concomitant infusion of vehicle *vs*. tolbutamide, an inhibitor of ATP-sensitive potassium channels in neurons, into the lateral ventricle. *In vitro*, we examined the direct effects of topiramate on glucose uptake and insulin signaling in C2C12 muscle cells.

Therapeutic concentrations of topiramate (~4 µg/ml) improved insulin sensitivity (glucose infusion rate +58%), which was the result of improved insulin-mediated glucose uptake by heart (+92%), muscle (+116%) and adipose tissue (+586%). Upon infusion of tolbutamide this insulin-sensitizing effect of topiramate was completely abrogated in hyperinsulinemic-euglycemic clamp conditions. Topiramate did not alter glucose uptake or insulin signaling in normal and insulin-resistant C2C12 muscle cells.

Topiramate stimulates insulin-mediated glucose uptake *in vivo* through the central nervous system. These observations illustrate the possibility of pharmacological modulation of peripheral insulin resistance through a target in the central nervous system.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a syndrome characterized by impaired insulin secretion in relation to decreased insulin sensitivity. Many drugs have been developed that act on these pathophysiological mechanisms. In recent years, evidence has accumulated that the central nervous system 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 central nervous system (1-4). Importantly, in insulin resistant conditions these effects of circulating insulin through the central nervous system are lost (1;4). The question arises whether the loss of these effects of insulin, mediated by the central nervous system, are amendable to pharmacological intervention.

Topiramate, a sulfamate-substituted derivative of the monosaccharide D-fructose (5), is used as an antiepileptic drug (6;7). The antiepileptic effects of topiramate are mediated through at least six mechanisms of action within the central nervous system (8-14). Studies in obese, diabetic rats demonstrated that topiramate treatment reduced plasma glucose levels and improved insulin sensitivity independently of weight loss (15;16). 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 central nervous system. Therefore, we studied in high-fat fed mice the effects of i.c.v. administered vehicle *vs.* 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 (1;3;4). In addition, we assessed the effects of topiramate in C2C12 muscle cells in order to evaluate the possibility of direct effects of topiramate on insulin signaling and glucose uptake. In this study we show that topiramate improves peripheral insulin sensitivity at least in part by improving insulin sensitivity in the brain.

MATERIALS AND METHODS

Animals

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, US), which has previously been shown to induce insulin resistance (17). 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, The Netherlands) with or without 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.

I.c.v. cannula implantation

For i.c.v. cannula implantation, 15-week-old male mice were anaesthetized with 0.5 mg/kg Medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands) and 0.05 mg/kg 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) and the anesthesia was antagonized using 2.5 mg/kg Antipamezol (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 mg/kg Flumazenil (Roche, Mijdrecht, The Netherlands) and 1.2 mg/kg 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, US) were considered to have the cannula correctly placed and were included in the study (1;18).

Hyperinsulinemic-euglycemic clamp studies

Overnight fasted, body weight-matched male mice were anaesthetized with 6.25 mg/kg Acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). First, basal rates of glucose turnover were determined by administration of a primed continuous intravenous (i.v.) infusion of D-[1-^{14}C]glucose (0.3 µCi/kg/min; Amersham, Little Chalfont, UK) for 60 minutes. Subsequently, insulin (Actrapid, Novo Nordisk, Denmark) was administered i.v. by primed (4.1 mU), continuous (6.8 mU/h) infusion to attain steady-state hyperinsulinemia together with D-[1-^{14}C]glucose (0.3 µCi/kg/min; Amersham, Little Chalfont, UK) 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, Little Chalfont, UK) 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 and after perfusion, organs were harvested and snap-frozen in liquid nitrogen.

I.c.v. tolbutamide treatment during clamp

As of thirty minutes before the start of the hyperinsulinemic-euglycemic clamp, aCSF or the K_{ATP} channel blocker tolbutamide, 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/h (3;19).

Plasma analysis

Blood samples were taken from the tail tip into chilled paraoxon-coated capillaries to prevent *ex vivo* lipolysis. The tubes were 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) in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Plasma insulin levels were

measured using a mouse-specific insulin ELISA kit (Crystal Chem Inc., Downers Grove, U.S.). Total plasma ^{14}C-glucose and ^{3}H-glucose were determined in supernatant of 7.5 µl plasma, after protein precipitation using 20% trichloroacetic 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 (20-22)) 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 Dulbecco's modified Eagle's Medium (DMEM) containing 25 mM glucose, glutamine and pyruvate (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). For deoxyglucose uptake assay ~15, 000 cells/well were seeded and cultured on 12-well plates (Greiner Bio-One). For Western blot analysis ~25, 000 cells/well were seeded and cultured on 6-well plates (Greiner Bio-One). When 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 (HS) instead of 10% FBS). The differentiation medium was changed every 48 h. The myotubes were used for experiments at day 7 of differentiation.

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 bovine serum albumin (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.

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 NaCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$ 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, Missouri, US) for 10 min in the presence of topiramate or vehicle. 2-Deoxy-D-[1-^{14}C] glucose was added to the cells for another 10 min (0.012 µCi/dish). The reaction was ended by washing three times with ice-cold PBS and addition of a lysis buffer containing 1% SDS/0.2 M NaOH. The lysates were transferred into plastic vials, 2 ml of scintillation liquid (Instagel Plus, PerkinElmer, Waltham, Massachusetts, US) was added and radioactivity was measured in a scintillation counter.

Western blot analysis

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 TrisPO4, pH 6.8. The cell lysates were then immediately boiled for 5 min and stored at -20°C until use. Protein content was determined using the bicinchoninic acid protein assay (Pierce, Rockford, Illinois, US). 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 phospho-Ser473-PKB antibody or a mix of PKBα and PKBβ antibodies (all from Cell Signaling Technology, Beverly, US). 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 (μmol/min/kg) were calculated in basal and hyperinsulinemic conditions as the rate of tracer infusion (dpm/min) divided by plasma specific activity of ^{14}C-glucose (dpm/μmol). 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 hyperinsulinemic-euglycemic clamp conditions.

Tissue-specific glucose uptake in heart, skeletal muscle and adipose tissue was calculated from tissue 2-[^{3}H]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 nonparametric 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 (26.6 ± 0.5 *vs.* 26.7 ± 0.6 g) and fasting plasma glucose levels (4.2 ± 0.2 *vs.* 4.3 ± 0.2 mmol/l) 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 (23), was 3.7 ± 0.2 μg/ml. After 6 weeks of topiramate treatment, the topiramate concentration in the brains of overnight fasted mice was 115 ± 18 ng/g brain tissue.

Effect of topiramate on insulin sensitivity in mice

After 6 weeks of topiramate treatment, insulin sensitivity was assessed using hyperinsulinemic-euglycemic clamp studies. Body weight did not differ between topiramate and vehicle treated mice (table 1). In the basal period of the hyperinsulinemic-euglycemic clamp, endogenous glucose production (EGP), which equals glucose disposal (Rd), was not different between topiramate and vehicle treated animals.

Table 1. Results obtained during the hyperinsulinemic-euglycemic clamp study in vehicle and topiramate treated animals. Data are represented as means ± SEM for at least 7 animals per group. There were no significant differences between any of the parameters.

	Vehicle	Topiramate
Body weight (g)	25.7 ± 0.4	24.9 ± 0.8
Basal hematocrit (%)	40.3 ± 0.2	38.5 ± 0.2
Clamp hematocrit (%)	36.8 ± 0.2	34.9 ± 0.5
Basal insulin (ng/ml)	0.5 ± 0.1	0.5 ± 0.1
Clamp insulin (ng/ml)	5.1 ± 0.4	4.3 ± 0.5
Basal glucose (mmol/l)	4.1 ± 0.1	4.2 ± 0.1
Clamp glucose (mmol/l)	4.5 ± 0.3	4.0 ± 0.2

The glucose infusion rate (GIR) necessary to maintain euglycemia was significantly higher in topiramate treated animals compared to vehicle treated animals (average GIR 84 ± 8 *vs*. 56 ± 4 µmol/min/kg for the last 20 min of the experiment, $P < 0.05$, Fig. 1A and Fig. 2B), in the presence of similar glucose levels (table 1 and Fig. 2A), indicating that topiramate improved insulin sensitivity. The specific activity of ^{14}C-glucose measured at 10 min intervals indicated the presence of steady-state conditions in all groups (table 2).

Table 2. Specific activity of ^{14}C-glucose (dpm/mmol) in the basal period or in the hyperinsulinemic euglycemic period in vehicle and topiramate treated animals. Values are means ± SEM for at least 7 mice per group. There were no significant differences between vehicle *vs*. topiramate treated animals.

	Basal period		Hyperinsulinemic euglycemic period		
	-10 min	0 min	70 min	80 min	90 min
Vehicle	$6.7 \pm 0.6 \times 10^3$	$6.2 \pm 0.6 \times 10^3$	$6.8 \pm 0.4 \times 10^3$	$6.2 \pm 0.5 \times 10^3$	$6.3 \pm 0.4 \times 10^3$
Topiramate	$6.6 \pm 0.5 \times 10^3$	$7.4 \pm 0.6 \times 10^3$	$5.3 \pm 0.3 \times 10^3$	$5.1 \pm 0.2 \times 10^3$	$5.0 \pm 0.3 \times 10^3$

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

sensitivity. 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) (Fig. 1D).

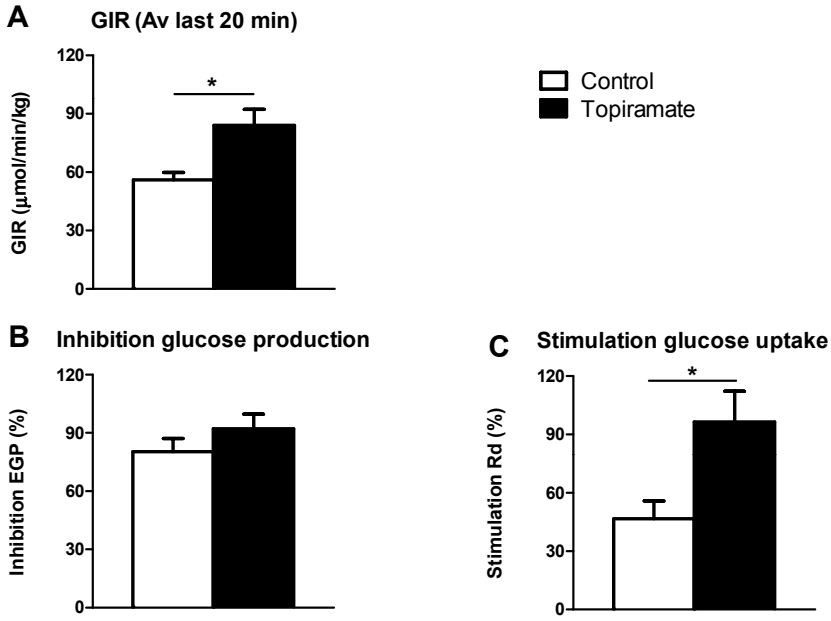

Fig. 1. Glucose infusion rate (GIR, A), endogenous glucose production (EGP, B), glucose disposal (Rd, C) in vehicle treated (white) and topiramate treated (black) animals as measured in hyperinsulinemic-euglycemic clamp studies. Values represent means ± SEM for at least 7 animals per group. *P < 0.05 *vs.* vehicle. Av, average.

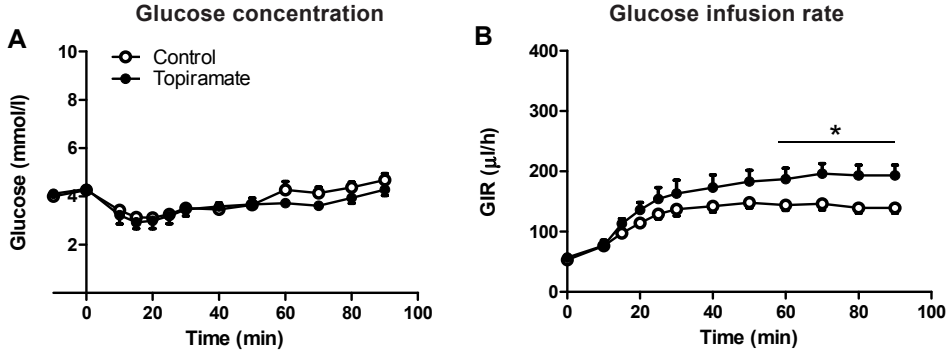

Fig. 2. Glucose concentrations (A) and glucose infusion rates (GIR, B) in vehicle treated (white) and topiramate treated (black) animals as measured in hyperinsulinemic-euglycemic clamp studies. Values represent means ± SEM for 7-10 mice per group. *P < 0.05, Topiramate *vs.* control.

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 signaling in the brain, tolbutamide, an inhibitor of ATP-sensitive potassium (K_{ATP}) channels in neurons, or vehicle was infused into the lateral ventricle (i.c.v.) during hyperinsulinemic-euglycemic clamp experiments. 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/kg for the last 20 min of the experiment, $P < 0.05$, Fig. 3A and Fig. 4B), in the presence of similar glucose levels (table 3 and Fig. 4A). I.c.v. tolbutamide administration in vehicle treated animals did not affect GIR compared to i.c.v. aCSF (66 ± 8 *vs*. 55 ± 11 µmol/min/kg for the last 20 min of the experiment, ns). I.c.v. administration of tolbutamide decreased GIR compared to topiramate treated animals receiving aCSF (average GIR 75 ± 7 *vs*. 85 ± 9 µmol/min/kg for the last 20 min of the experiment, $P < 0.05$), indicating that i.c.v. tolbutamide counteracted, at least in part, the improvement in insulin sensitivity induced

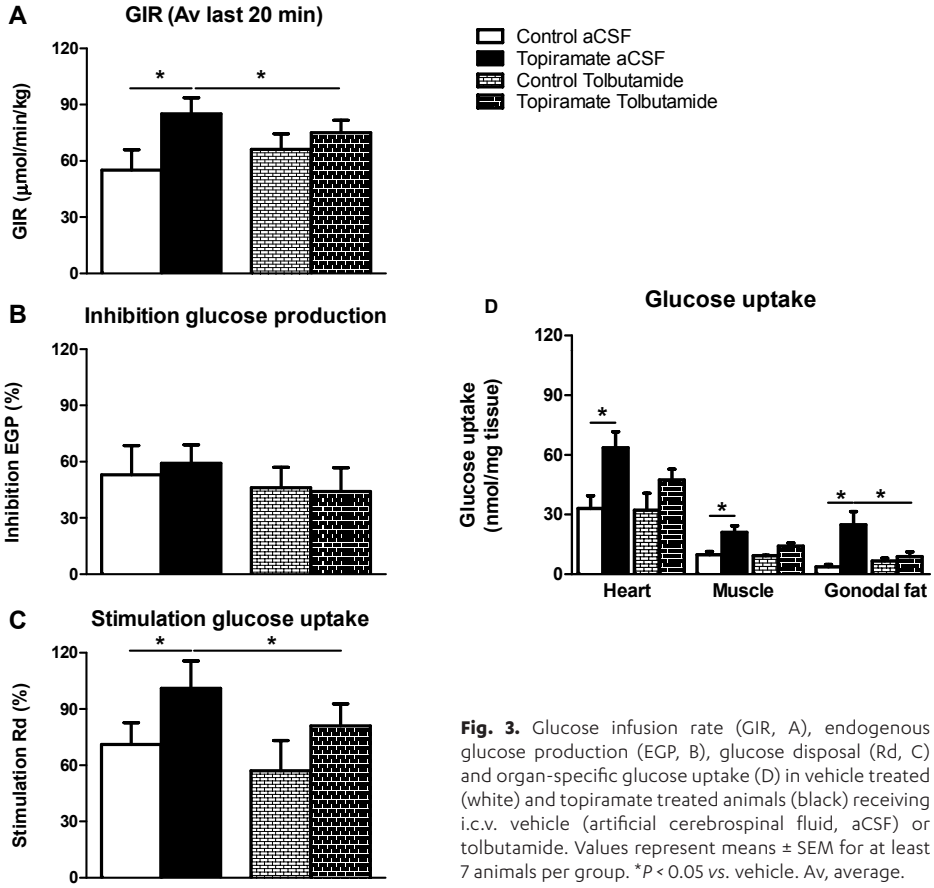

Fig. 3. Glucose infusion rate (GIR, A), endogenous glucose production (EGP, B), glucose disposal (Rd, C) and organ-specific glucose uptake (D) in vehicle treated (white) and topiramate treated animals (black) receiving i.c.v. vehicle (artificial cerebrospinal fluid, aCSF) or tolbutamide. Values represent means ± SEM for at least 7 animals per group. *$P < 0.05$ *vs*. vehicle. Av, average.

by topiramate. The specific activity of ^{14}C-glucose measured at 10 min intervals indicated the presence of steady-state conditions in all groups (table 4). In the basal period as well as in the hyperinsulinemic period, EGP was not different between all groups of mice (Fig. 3B). Insulin-stimulated Rd was again significantly higher in topiramate treated animals compared to vehicle treated animals receiving aCSF (101 ± 15 *vs*. 71 ± 12 %, respectively, $P < 0.05$, Fig. 3C). I.c.v. tolbutamide administration in vehicle treated animals did not affect Rd compared to i.c.v. aCSF (57 ± 16 *vs*. 71 ± 12 µmol/min/kg for the last 20 min of the experiment, ns). However, i.c.v. administration of tolbutamide in topiramate animals diminished the topiramate-improved Rd (81 ± 12 *vs*. 101 ± 15 %, respectively, $P < 0.05$). In accordance, topiramate treated animals had higher 2-[^{3}H]DG uptake in heart, skeletal muscle and adipose tissue compared to vehicle

Table 3. Results of i.c.v. administration of aCSF and tolbutamide obtained from the hyperinsulinemic-euglycemic clamp study in vehicle and topiramate treated animals. Data are represented as means ± SEM for at least 8 animals per group. There were no significant differences between vehicle *vs*. topiramate treated animals. 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 hematocrit (%)	40.3 ± 0.2	39.0 ± 0.8	38.5 ± 0.2	37.1 ± 0.3
Clamp hematocrit (%)	36.8 ± 0.2	36.3 ± 0.2	34.9 ± 0.5	34.4 ± 0.4
Basal insulin (ng/ml)	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Clamp insulin (ng/ml)	4.1 ± 0.4	3.3 ± 0.1	3.4 ± 0.2	3.8 ± 0.4
Basal glucose (mmol/l)	5.0 ± 0.2	5.4 ± 0.3	5.5 ± 0.2	4.9 ± 0.2
Clamp glucose (mmol/l)	5.6 ± 0.3	6.0 ± 0.5	5.8 ± 0.3	5.2 ± 0.3
Basal FFA (mmol/l)	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0
Clamp FFA (mmol/l)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

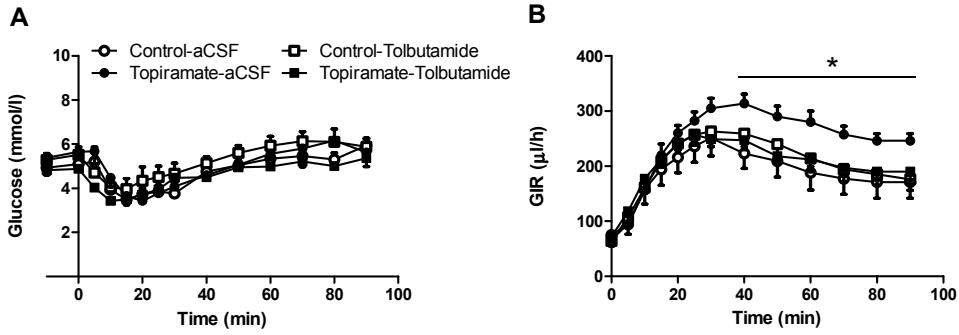

Fig. 4. Glucose concentrations (A) and glucose infusion rates (GIR, B) in vehicle treated (white) and topiramate treated animals (black) receiving i.c.v. vehicle (aCSF) or tolbutamide. Values represent means ± SEM for 7-10 mice per group. *$P < 0.05$, Topiramate *vs*. other groups.

treated animals (Fig. 3D). In line with Rd, i.c.v. tolbutamide did not affect 2-[^{3}H]DG uptake compared to i.c.v. aCSF in vehicle treated animals. 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.

Table 4. Specific activity of ^{14}C-glucose (dpm/mmol) in the basal period or in the hyperinsulinemic euglycemic period in vehicle and topiramate treated animals. Values are means ± SEM for at least 7 mice per group. There were no significant differences between vehicle *vs.* topiramate treated animals.

	Basal period		**Hyperinsulinemic euglycemic period**		
	-10 min	**0 min**	**70 min**	**80 min**	**90 min**
Vehicle aCSF	7.2 ± 1.0 x 10^3	7.7 ± 0.7 x 10^3	4.3 ± 0.5 x 10^3	4.4 ± 0.4 x 10^3	4.4 ± 0.4 x 10^3
Topiramate aCSF	5.8 ± 0.5 x 10^3	6.0 ± 0.5 x 10^3	3.3 ± 0.2 x 10^3	3.1 ± 0.2 x 10^3	3.2 ± 0.3 x 10^3
Vehicle Tolbutamide	6.7 ± 0.9 x 10^3	6.9 ± 0.6 x 10^3	3.5 ± 0.3 x 10^3	3.3 ± 0.4 x 10^3	3.8 ± 0.3 x 10^3
Topiramate Tolbutamide	7.9 ± 1.0 x 10^3	8.0 ± 0.9 x 10^3	3.9 ± 0.2 x 10^3	4.4 ± 0.6 x 10^3	3.9 ± 0.3 x 10^3

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 (Fig. 5A). 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, i.e. without topiramate. Topiramate did not increase basal or insulin-stimulated glucose uptake (Fig. 5A).

Effect of topiramate on insulin signaling in differentiated myotubes

Western blot analyses were performed to determine whether topiramate affects the insulin signaling pathway in differentiated myotubes by assessing the phosphorylation state of Akt/PKB (Fig. 5B). As expected, insulin increased the phosphorylation state of Akt/PKB on Ser^{473} by +583% in control condition. Topiramate did not affect basal or insulin-stimulated phosphorylation of Akt/PKB (Fig. 5B).

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 (Fig. 6). After incubation with palmitate for 16 h, C2C12 myotubes were incubated with increasing concentrations of topiramate (1 or 100 µM) for 45 minutes and challenged with or without 1 µM of insulin for 20 min. At maximal concentration of insulin, glucose uptake was increased by +25% in control condition, i.e. without palmitate and topiramate (Fig. 6A). 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 (Fig. 6B, C).

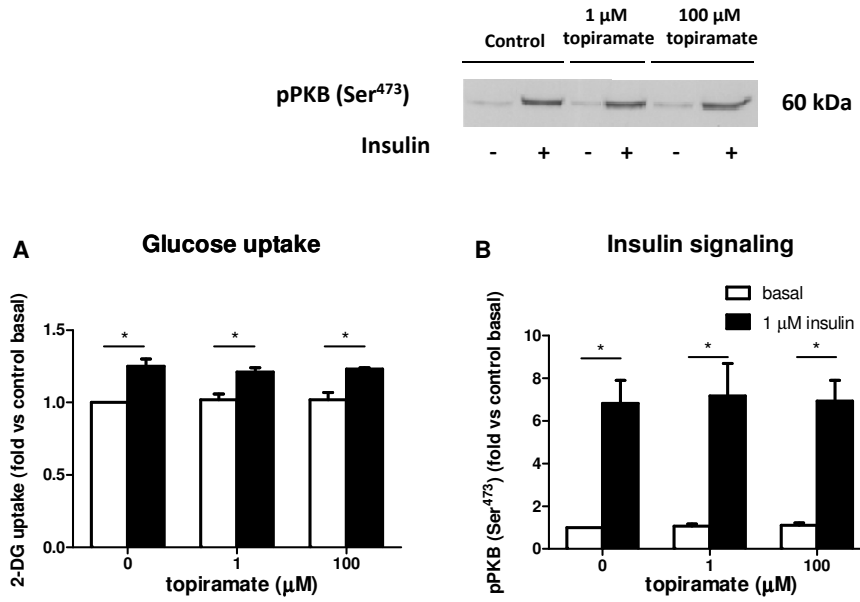

Fig. 5. Glucose uptake (A) and insulin signaling (B) in C2C12 myotubes at basal (white) or insulin-stimulated (black) condition with topiramate (1 or 100 µM) or vehicle. The quantification was normalized for total protein and expressed as fold change compared to vehicle at basal condition. Data are represented as means ± SEM for n=3-4, * $P < 0.05$ *vs*. vehicle.

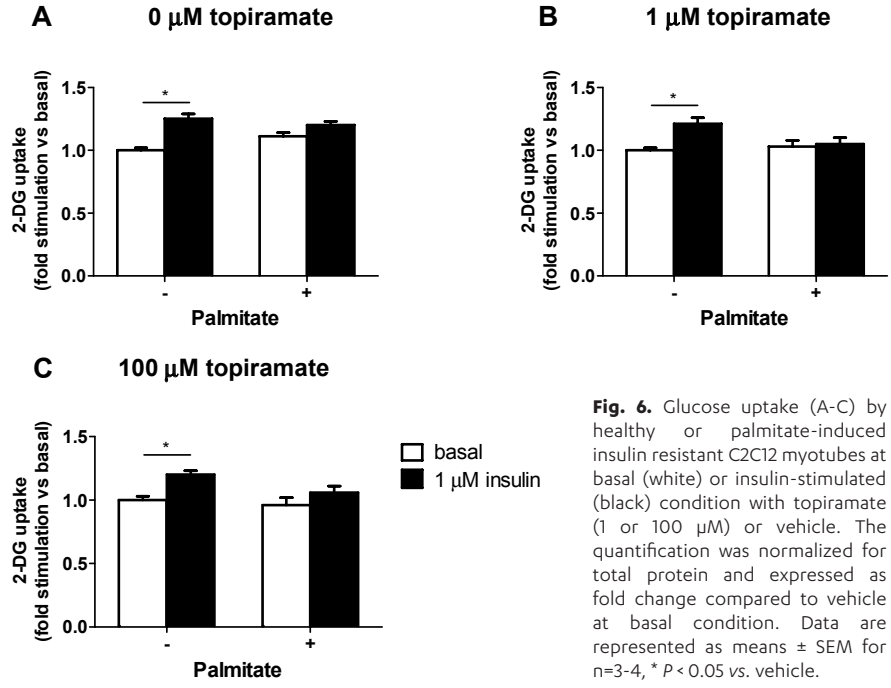

Fig. 6. Glucose uptake (A-C) by healthy or palmitate-induced insulin resistant C2C12 myotubes at basal (white) or insulin-stimulated (black) condition with topiramate (1 or 100 µM) or vehicle. The quantification was normalized for total protein and expressed as fold change compared to vehicle at basal condition. Data are represented as means ± SEM for n=3-4, * $P < 0.05$ *vs*. vehicle.

DISCUSSION

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, 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. *In vitro*, topiramate had no direct effect on basal or insulin-stimulated glucose uptake. Collectively, these data indicate that topiramate improves peripheral insulin sensitivity indirectly via the brain, rather than directly, in 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 resistent subjects (24). The half-life of topiramate in humans is 21 h, whereas the half-life in rodents is only 1-2 h (25). 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 bitter taste. The dose of topiramate used in the present study resulted in concentrations in plasma within the therapeutic range (~4 µg/ml). Hyperinsulinemic-euglycemic clamp analyses revealed that topiramate improved insulin sensitivity, in agreement with previous studies. Those studies indicated that topiramate improved insulin sensitivity independent of weight loss (16;26;27). 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 (26). As α-adrenergic stimulation enhances AMPK phosphorylation, the increased glucose uptake by peripheral organs might be related to increased sympathetic nervous system (SNS) activation (28). Furthermore, the topiramate concentration in plasma correlates with that in cerebral spinal fluid (CSF) (29). Moreover, the effects of topiramate on body weight, body composition and energy metabolism have been associated with altered neuropeptide expression in the hypothalamus (30). Combined with the absence of direct effects of topiramate on insulin sensitivity in muscle cells, we therefore hypothesized that the brain mediated the effects of topiramate on insulin sensitivity.

The indirect effects of circulating insulin, through the central nervous system, on peripheral glucose uptake are blocked by i.c.v. administration of tolbutamide (1;3). Insulin activates ATP-sensitive potassium channels (K_{ATP} channels) in neurons of the hypothalamus (31;32). I.c.v. administration of tolbutamide inhibits activation of these neuronal K_{ATP} channels by insulin (1;3). To test our hypothesis that the central nervous system is involved in the anti-diabetic effects of topiramate by improving insulin signaling in the brain, we administered tolbutamide i.c.v. in topiramate-treated animals during hyperinsulinemic-euglycemic clamp conditions. Interestingly, i.c.v. administration of tolbutamide abolished the improvement of insulin sensitivity by topiramate during clamp conditions. In other words, the insulin-sensitizing effect of topiramate apparently originates from an insulin sensitization effect in the brain. In previous studies, we showed that high-fat feeding results in insulin resistance in the brain (1;4). 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. I.c.v. tolbutamide in topiramate treated animals abolished the insulin-sensitizing effect of topiramate, suggesting that the brain mediates the effects of topiramate on insulin sensitivity.

Topiramate did not exert any direct effects on basal or insulin-stimulated glucose uptake and insulin signaling in insulin-sensitive or insulin-resistant C2C12 myotubes. 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 AMP-activated protein kinase (AMPK)-mediated pathway (33). Treatment of C2C12 myotubes with topiramate did not increase phosphorylation level of AMPK (Thr172) (data not shown). Stimulation of a rat insulinoma-derived INS-1E cell line with topiramate did not affect glucose-stimulated insulin secretion by these beta cells (data not shown), in line with a previous study (34). Our data support the notion that the effects of topiramate on insulin sensitivity are most likely not the result of direct effects on peripheral organs.

In conclusion, topiramate improves insulin sensitivity in high-fat fed mice by stimulating glucose uptake by skeletal muscle, heart and adipose tissue through effects within the central nervous system. 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 action in the brain, rather than direct effects of topiramate on peripheral organs. These observations illustrate the possibility of pharmacological treatment of peripheral insulin resistance through targets in the central nervous system.

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