

Topiramate stimulates glucose transport through AMP-activated protein kinase-mediated pathway in L6 skeletal muscle cells

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The use of topiramate (TPM) in the treatment of binge-eating disorder, bulimia nervosa, and antipsychotic-induced weight gain has recently increased, however, the exact molecular basis for its effects on body weight reduction and improved glucose homeostasis, is yet to be elucidated. Here we investigated the effect and signaling pathway of TPM on glucose uptake in L6 rat skeletal muscle cells, which account for >70% of glucose disposal in the body. Intriguingly, we found that TPM (10 μ M) stimulated the rate of glucose uptake up to twofold increase. And TPM-stimulated glucose transport was inhibited with the overexpression of dominant-negative form of AMP-activated protein kinase (AMPK), an important mediator in glucose transport, implicating that AMPK-mediated pathway is involved. The TPM-stimulated glucose transport was blocked by SB203580, a specific inhibitor of AMPK downstream mediator, p38 mitogen-activated protein kinase (MAPK) protein. LY294002, an inhibitor of phosphatidylinositol (PI) 3-kinase, which is another crucial mediator in independent glucose transport pathway, did not inhibit TPM-stimulated glucose transport. We also found that TPM increased the phosphorylation level of AMPK and p38 MAPK, whereas no effect on the activity of PI 3-kinase of TPM, when assessed by PI 3-kinase assay, was observed. These results together suggest that TPM stimulates glucose transport, not via PI 3-kinase mediated, but via AMPK-mediated pathway in skeletal muscle cells, thereby contributing to the body weight regulation and glucose homeostasis.

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Introduction

Topiramate (TPM) is an antiepileptic agent and potentially has additional psychiatric applications, including bipolar disorder, migraine, and neuropathic pain.¹ Promising evidences for the use of TPM as an effective treatment for binge-eating disorder and bulimia nervosa have been provided recently.^{2–6} In a case report of bipolar disorder patient with obesity-related type 2 diabetes mellitus, TPM treatment was associated with body weight loss and improvement of glucose homeostasis.⁷ TPM has also been reported to reduce fat gains.¹

Even though the atypical antipsychotics with the therapeutic benefits have been used in the treatment of psychiatric disorders, especially schizophrenia, intense scientific interest and growing public concern regarding the risk of developing weight gain, diabetes mellitus, and related metabolic disturbances are emerging.^{8–12} Statistically significant increases in weight gain were seen in patients treated with clozapine or quetiapine.¹³ Consistent finding which reported higher rate of diabetes with a particular emphasis on clozapine, olanzapine, and quetiapine was also reported.¹³ Studies also implicate an association between antipsychotics medication and glucose dysregulation.^{14,15} As the incidence of eating disorders and antipsychotic-induced weight gain have been increasing for the past decade, body weight reduction and improved glucose homeostasis by TPM treatment are of particular interest.

Thus we set out the study to test the hypothesis that TPM might play a role in the process of glucose transport and to investigate whether AMP-activated protein kinase (AMPK) or phosphatidylinositol (PI) 3-kinase-mediated glucose transport pathway is involved in the process in L6 rat skeletal muscle cells.

Results

TPM stimulates glucose transport in skeletal muscle cells

We determined the effect of TPM on glucose transport using 2-[³H] deoxy-D-glucose in L6 skeletal muscle cells. Figure 1 shows that glucose uptake by TPM was increased ~200% compared to control ($P < 0.01$). TPM showed dose-independent stimulatory effect on glucose uptake. At the concentrations of 10, 100, and 1000 μM , TPM-stimulated glucose transports were 192 ± 13.9 , 191 ± 23.6 , and $199 \pm 22.1\%$, respectively. The stimulatory effect of TPM on glucose uptake was greater than that of insulin (10 nM) ($148 \pm 15.8\%$), which was employed as a positive control.

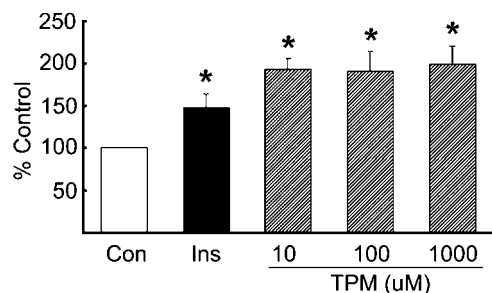


Figure 1 Topiramate stimulates the rate of 2-[³H] deoxy-D-glucose uptake in L6 skeletal muscle cells. Cells were treated with various concentrations of topiramate (10–1000 μM) for 30 min and 2-[³H] deoxy-D-glucose uptake was determined as described under Materials and methods. Insulin (10 nM) was used as a positive control. Data represent the mean \pm standard error ($n=6$) of two separate experiments, each performed in triplicate and presented as percentage increase. * $P < 0.01$ vs control. Con, control; Ins, insulin; TPM, topiramate.

This result suggests that TPM stimulates glucose transport in L6 skeletal muscle cells.

As TPM showed dose-independent effect on glucose transport and 10 μM TPM was employed in rat primary astroglial cells,¹⁶ we used 10 μM TPM throughout the experiment.

TPM-stimulated glucose transport is mediated via AMPK and p38 MAPK

AMPK and PI 3-kinase are two major important mediators in the regulation of two independent pathways involved in glucose transport. Therefore, we examined the possible involvement of these pathways in TPM-stimulated glucose transport in L6 skeletal muscle cells using specific inhibitors of p38 MAPK (10 μM SB203580) and PI 3-kinase (20 μM LY294002). Insulin (10 nM) was employed as a positive control. As shown in Figure 2, TPM increased the rate of glucose uptake ~twofold ($182 \pm 14.8\%$, $P < 0.01$) compared to control and showed stronger stimulatory effect on glucose transport than insulin ($147 \pm 15.3\%$). SB203580, but not LY294002, significantly inhibited TPM-stimulated glucose transport ($125 \pm 19.3\%$). TPM-stimulated glucose transport was decreased by SB203580 ~70% compared to the increased rate of glucose transport by TPM ($P < 0.01$). This result indicates that p38 MAPK is involved in the stimulatory effect of TPM on glucose transport. LY294002 showed no inhibitory effect on TPM-stimulated glucose transport ($169 \pm 13.8\%$). As AMPK activates p38 MAPK,^{17,18} we then examined the involvement of AMPK in the regulation of TPM-stimulated glucose transport by transiently transfecting L6 cells with dominant-negative AMPK containing pcDNA3 plasmids. Glucose transport was greatly increased by TPM ($201 \pm 18.0\%$). Transfection of dominant-negative AMPK, however, decreased the TPM-stimulated glucose transport by ~70% (201 ± 23.4 vs $131 \pm 23.8\%$,

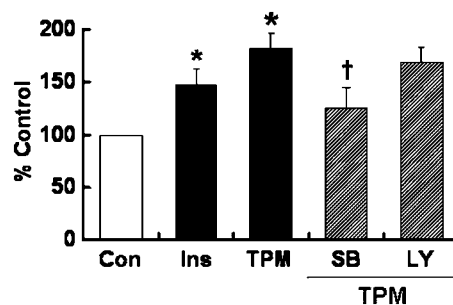


Figure 2 Effects of specific inhibitors of p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol (PI) 3-kinase on topiramate-stimulated glucose transport in skeletal muscle cells. Cells were treated with 10 μM topiramate for 30 min in the absence or presence of 10 μM SB203580 (p38 MAPK specific inhibitor), or 20 μM LY294002 (PI 3-kinase specific inhibitor) for 45 min and glucose uptake was measured as described under Materials and methods. Insulin (10 nM) was used as a positive control. Data represent the mean \pm standard error ($n=6$) of two separate experiments each performed in triplicate and presented as % increase. * $P < 0.01$ vs control, † $P < 0.01$ vs TPM-treated cells. Con, control; Ins, insulin; TPM, topiramate; SB, SB203580; LY, LY294002.

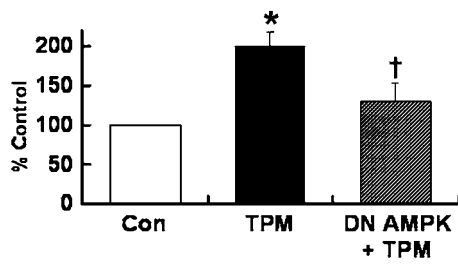


Figure 3 Effects of dominant-negative AMP-activated protein kinase on topiramate-stimulated glucose transport in skeletal muscle cells. Cells were seeded onto 24-well culture plates at a density of 4×10^4 cells/well and incubated for 24 h in media. The plasmids (dominant-negative AMPK or pcDNA3) were transfected into L6 cells. Data represent the mean \pm standard error ($n=6$) of two separate experiments each performed in triplicate and presented as percentage increase. * $P < 0.01$ vs control, † $P < 0.01$ vs TPM-treated cells. Con, control; TPM, topiramate; DN AMPK, dominant-negative AMP-activated protein kinase.

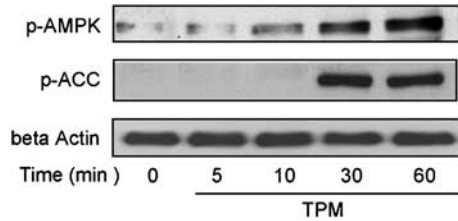


Figure 4 Effects of topiramate on the phosphorylation state of AMP-activated protein kinase. Cells were treated with $10 \mu\text{M}$ topiramate for the indicated time. Cell lysates ($50 \mu\text{g}$) were analyzed by immunoblotting with antibodies specific to phospho-AMPK at Thr¹⁷² (1:1000) and phospho-acetyl-CoA carboxylase at Ser⁷⁹ (1:1000). Beta actin was used as an internal control. TPM, topiramate; p-AMPK, phospho-AMP-activated protein kinase; p-ACC, phospho-acetyl-CoA carboxylase.

$P < 0.01$) (Figure 3). The decreased rate of glucose transport by transfection of dominant-negative AMPK was similar to the decreased rate of glucose transport by SB203580. The data confirm that TPM stimulated glucose transport through AMPK-mediated pathway.

TPM increases the phosphorylation levels of AMPK and p38 MAPK
To verify that TPM-stimulated glucose transport is mediated via AMPK, we then examined the phosphorylation levels of AMPK and p38 MAPK proteins. As shown in Figure 4, phosphorylation of AMPK at Thr¹⁷² was gradually increased until 10 min and starting 30 min after TPM treatment, the phosphorylation state of AMPK was greatly increased. There also was an increase in the phosphorylation level of ACC at Ser⁷⁹, a downstream target of AMPK and a good correlate of AMPK activation.^{19,20} The phosphorylation of ACC was not detected at 5 and 10 min. At 30 and 60 min, greatly increased phosphorylation of ACC was observed. In agreement with the expression pattern of AMPK, p38 MAPK phosphorylation at Thr¹⁸⁰ and Tyr¹⁸² was also increased (Figure 5). Phosphorylated p38 was

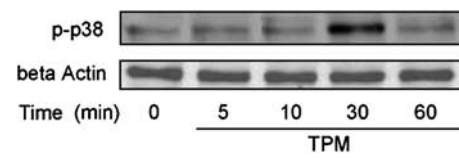


Figure 5 Effects of topiramate on the phosphorylation state of p38 mitogen-activated protein kinase. Cells were treated with $10 \mu\text{M}$ topiramate for the indicated time. Cell lysates ($50 \mu\text{g}$) were analyzed by immunoblotting with antibodies specific to phospho-p38 MAPK at Thr¹⁸⁰ and Tyr¹⁸² (1:1000). Beta actin was used as an internal control. TPM, topiramate; p-p38, phospho-p38.

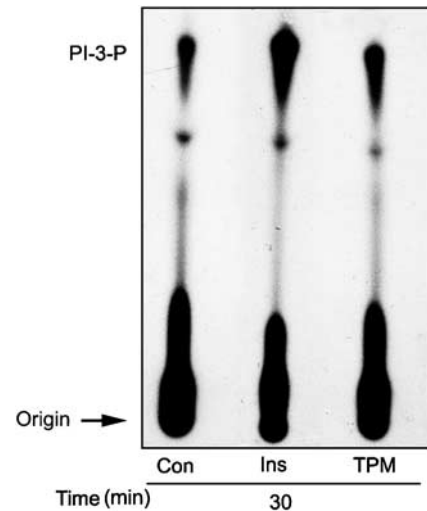


Figure 6 Effects of topiramate on phosphatidylinositol 3-kinase activation. Phosphatidylinositol 3-kinase enzymatic activity was measured in antiphosphotyrosine immunocomplexes as described under Materials and methods. Insulin (10 nM) was used as a positive control. Con, control; Ins, insulin; TPM, topiramate; PI-3-P, phosphatidylinositol-3-phosphate.

prominent at 30 min and decreased, however, at 60 min. The intensity of beta actin, which was used as an internal control, was consistent.

TPM-stimulated glucose transport is not mediated via PI 3-kinase
PI 3-kinase also plays a critical role in insulin-stimulated glucose transport. We performed PI 3-kinase assay using $[\gamma\text{-}^{32}\text{P}]$ ATP. In Figure 6, autoradiogram of a TLC plate shows the incorporation of ^{32}P into the 3' position of PI. Phosphatidylinositol-3-phosphate (PI-3-P), which is formed by PI 3-kinase activity, was markedly increased by insulin (10 nM). The level of PI-3-P by treatment of $10 \mu\text{M}$ TPM, however, was similar to that of control (Figure 6). Densitometry analysis revealed that insulin increased PI 3-kinase activity more than 200% ($209 \pm 3.4\%$) while TPM treatment slightly changed PI 3-kinase activity ($115 \pm 5.3\%$). This result is consistent with that in Figure 2, where treatment of PI 3-kinase specific inhibitor LY294002 did not affect TPM-stimulated glucose transport in skeletal muscle cells.

Discussion

Antipsychotic-induced weight gain is one of the leading causes that prohibit patients from continuing treatment. Recently, much interest has been focused on the weight loss effect of TPM, a generally well-tolerated antiepileptic drug. Evidence showed that TPM decreased food intake in obese Zucker rats.¹ TPM reduced blood glucose levels in both obese Zucker rats and db/db mice.²¹ It is also efficacious in the treatment of binge-eating disorder associated with obesity.²² Clinical trials of TPM in obese patients demonstrated that TPM was significantly more efficacious than placebo.^{5,6,22} Current investigation of TPM as a potential new therapy for obesity is underway.²³

Glucose uptake lies at the center when dealing with energy expenditure and glucose homeostasis. Skeletal muscle disposes off more than 70% of glucose in the body. Thus, glucose transport in skeletal muscle is a critical process in energy utilization.^{24,25} Among different signaling pathways that have been proposed in glucose transport, two independent signaling pathways are important to mediate glucose transport in response to various stimuli.²⁶ AMPK, mainly responsive to cell stress, is a critical regulatory protein in glucose transport pathway.^{27–29} It mediates contraction-induced glucose uptake in skeletal muscle.³⁰ Activation of AMPK stimulated p38 MAPK, implicating p38 MAPK is a downstream molecule of AMPK.

To identify the involved signaling pathway in TPM-stimulated glucose transport, we employed specific inhibitors, SB203580 and LY294002. Our data showed that AMPK mediates the stimulatory effect of TPM on glucose transport (Figure 2). This result is in line with that of the previous study showing that AMPK phosphorylation is upregulated in muscle in TPM-treated rats.³¹ Inhibition of AMPK by dominant-negative AMPK decreased the TPM-stimulated glucose uptake ~70%, further confirming that AMPK is involved in the stimulatory effect of TPM on glucose transport (Figure 3). Treatment of skeletal muscle cells with TPM also increased the phosphorylation levels of AMPK and p38 MAPK (Figures 4 and 5). PI 3-kinase represents another major switch element for AMPK-independent glucose transport pathway and is required for insulin-stimulated glucose uptake.²⁸ In this study, however, PI 3-kinase was not activated by TPM treatment, suggesting that PI 3-kinase is not involved in TPM-stimulated glucose transport in L6 skeletal muscle cells (Figure 6).

Our present study investigated the effect of TPM on glucose transport at the molecular level. To the authors' best knowledge, this is the first report showing that TPM, via AMPK-mediated pathway, stimulates the rate of glucose transport in skeletal muscle cells. We also showed that PI 3-kinase dependent pathway does not mediate TPM-stimulated glucose transport in skeletal muscle cells. As not much research to investigate the underlying mechanism of the effect of TPM on weight regulation and glucose homeostasis has been conducted, it is authors' hope that the present study will contribute to the future investigations.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA) unless otherwise indicated. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle medium (DMEM) and other culture products were purchased from GIBCO BRL (San Diego, CA, USA). LY294002 and SB203580 were obtained from Calbiochem (St Louis, MO, USA). [γ -³²P] ATP (6000 Ci/mmol) and 2-[³H] deoxy-D-glucose (6.0 Ci/mmol) were purchased from PerkinElmer Life And Analytical Sciences, Inc. (Boston, MA, USA). The antiphospho-specific antibodies that recognize a phosphoactivated form of AMPK, p38, and an antibody recognizing the phosphorylated acetyl-CoA carboxylase (ACC) Ser⁷⁹ were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Monolayer of L6 skeletal muscle cells were maintained at subconfluent conditions in growth media containing DMEM with 4.5 g/l glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal bovine serum. Cells were grown in a humidified, 37°C incubator with ambient oxygen and 5% CO₂.

Glucose uptake assay

Glucose uptake was essentially determined as previously described.³² Cells were cultured on 12-well culture plates, washed with Krebs–Ringer phosphate buffer (KRB) (pH 7.4, 25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 5 mM NaHCO₃, 0.07% bovine serum albumin, and 5.5 mM glucose) and incubated in KRB buffer for 60 min. Cells were then exposed to experimental reagents for 30 min. Glucose uptake was measured by adding 20 μ l glucose mixture (5 mM 2-deoxyglucose and 0.5 μ Ci 2-[³H] deoxy-D-glucose in KRB) to 980 μ l KRB and incubating for 20 min at 37°C. Nonspecific glucose uptake was measured by parallel incubations in the presence of 10 μ M cytochalasin B, which blocks transporter mediated glucose uptake, and was subtracted from total uptake in each assay. Uptake was terminated by washing the cells three times with 1 ml ice-cold phosphate-buffered saline. Cells were subsequently lysed with 0.5 ml of 0.5 M NaOH solution containing 0.1% SDS, and the solution was rotated for 15 min. Cell-associated radioactivity was measured by a liquid scintillation counter (PerkinElmer Life And Analytical Sciences, Inc., Boston, MA, USA).

PI 3-kinase assay

PI 3-kinase activity was measured by immunoprecipitation with antiphosphotyrosine antibody.³³ Cell lysates (500 μ g protein) were incubated with 20 μ l antiphosphotyrosine antibody agarose beads. After washing, the immunoprecipitates with PI 3-kinase activity were resuspended in 100 μ l kinase assay buffer containing 10 μ Ci [γ -³²P]

ATP and incubated for 30 min at room temperature with constant shaking. PI 3-K activity was measured by the phosphorylation of PI. The reaction was stopped by the addition of 100 μ l 1 M HCl and the reaction products were extracted with 200 μ l chloroform:methanol (1:1). The samples were centrifuged and the lower organic phase was harvested and applied to a silica gel thin layer chromatography (TLC) plate (Merk, Aichach, Germany) coated with 1% potassium oxalate. TLC plates were developed in chloroform:methanol:ammonium hydroxide: water (120:94:23.2:4), dried, and visualized by autoradiography.

Transient transfection

Cells were seeded onto 24-well culture plates at a density of 4×10^4 cells/well and incubated for 24 h in media. Plasmids (dominant-negative AMPK or pcDNA3) were transfected into cells using GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA, USA) according to the manufacturer's instructions. Dominant-negative AMPK form containing pcDNA3³⁴ was a generous gift from Dr Joohun Ha from Kyung Hee University, Seoul, Republic of Korea. Controls were transfected with pcDNA3 plasmid. After 24 h of transfection, glucose transport assay was performed. At least two independent transfections were performed in triplicate.

Immunoblotting

The cell lysates were centrifuged for 15 min at 12 000g at 4°C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell, Middlesex, UK). After transfer, the membrane was blocked in 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 (TBS-T) and then was incubated for 1 h at room temperature with primary antibody. Next, membrane was washed in TBS-T followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia, Uppsala, Sweden).

Statistical analysis

Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (one-way-ANOVA). The accepted level of significance was preset as *P*-value < 0.05. Data are presented as means \pm standard error (s.e.). The SAS Statistical Software Package (release 8.02; SAS Institute Inc., Cary, NC, USA) was used.

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Duality of interest

None declared.

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