Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues

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LeBrasseur, Nathan K., Meghan Kelly, Tsu-Shuen Tsao, Stephen R. Farmer, Asish K. Saha, Neil B. Ruderman, and Eva Tomas. Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. Am J Physiol Endocrinol Metab 291: E175-E181, 2006. First published February 7, 2006; doi:10.1152/ajpendo.00453.2005.—Thiazolidinediones (TZDs) are insulin-sensitizing agents used in the treatment of type 2 diabetes. A widely held view is that their action is secondary to transcriptional events that occur when TZDs bind to the nuclear receptor PPARγ in the adipocyte and stimulate adipogenesis. It has been proposed that this increases insulin sensitivity, at least in part, by increasing the expression and release of adiponectin, an adipokine that activates the fuel-sensing enzyme AMP-activated protein kinase (AMPK). In this study, we report that TZDs also acutely activate AMPK in skeletal muscle and other tissues by a mechanism that is likely dependent on PPARγ-regulated gene transcription. Thus incubation of isolated rat EDL muscles in medium containing 5 mM troglitazone for 15 min (too brief to be attributable to transcription) significantly increased pAMPK and pACC. At a concentration of 100 μM, troglitazone maximally increased these parameters and caused twofold increases in 2-deoxy-D-glucose uptake and the oxidation of exogenous [14C]palmitate. Time course studies revealed that troglitazone-induced increases in pAMPK and pACC abundance at 15 min were paralleled by an increase in the AMP-to-ATP ratio and that by 60 min all of these parameters had returned to baseline values. Increases in pAMPK and pACC were also observed in skeletal muscle, liver, and adipose tissue in intact rats 15 min after the administration of a single dose of troglitazone (10 mg/kg, ip). Likewise, troglitazone and another TZD, pioglitazone, caused rapid increases in pAMPK and pACC of equal magnitude in Swiss 3T3 fibroblasts with and without sufficient PPARγ to mediate the expression of target genes. The results indicate that TZDs can act within minutes to activate AMPK in mammalian tissues. They suggest that this effect is associated with a change in cellular energy state and that it is not dependent on PPARγ-mediated gene transcription.

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(p)AMPK (Thr\textsuperscript{172}) were acquired from Cell Signaling Technology (Beverly, MA). A polyclonal antibody that immunoprecipitates the α2 catalytic subunit of AMPK and was used for activity assays was generated by Quality Control Biochemicals (Hopkinton, MA). Phospho-acetyl-CoA carboxylase (pACC) (Ser\textsuperscript{79}) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Protein A/G sepharose beads, actin, PPAR\textgamma, and mouse, rabbit, and goat antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). We acquired 2-deoxy-o-[1,2-\textsuperscript{3}H]-glucose (2-DG), [U-\textsuperscript{14}C]mannitol, and [U-\textsuperscript{14}C]palmitate from NEN Research Products (Boston, MA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA), and cell lysis buffer was obtained from Cell Signaling. All other chemicals were of analytical grade.

Animals. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Boston University Medical Center and were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (50–65 g) were purchased from Charles River Breeding Laboratories. Animals were maintained on a 12/12-h light-dark cycle in a temperature-controlled (19–21°C) room and were fed standard rodent chow and water ad libitum. Food was withdrawn 16–20 h before the initiation of experimental protocols.

Muscle incubation studies. Intact extensor digitorum longus (EDL) muscles were dissected, sutured to stainless steel clips, and incubated in Krebs-Henseleit medium as previously described (39). Prior to experimental protocols, muscles were equilibrated in oxygenated (95% O\textsubscript{2}–5% CO\textsubscript{2}) Krebs-Henseleit solution containing 6 mM glucose for 20 min at 37°C. Dose response experiments were conducted in medium containing 0–250 μM troglitazone for 15 min, and for time-course studies muscles were incubated with 0, 5, or 100 μM troglitazone for 5, 15, 30, or 60 min. To measure glucose transport, EDLs were treated for 30 min with or without troglitazone (100 μM), insulin (10 nM/ml), or troglitazone plus insulin in the presence of 0.3 μCi/ml 2-DG (11.8 nM 2-DG–5.5 mM glucose) and 0.06 μCi/ml [U-\textsuperscript{14}C]mannitol (as an extracellular space marker) (23). Fatty acid oxidation was assessed on the basis of 14CO\textsubscript{2} release into the medium in muscle incubated with or without troglitazone (100 μM) and 0.2 μCi/ml [U-\textsuperscript{14}C]palmitate (20). At the end of the incubation protocols, muscles were blotted, quick-frozen in liquid nitrogen, and stored at −80°C until analyses were performed.

Cell culture and PPAR\textgamma overexpression. Swiss-3T3 cells expressing either retroviral PPAR\gamma (3T3-PPAR\gamma) or control vector (3T3-Con) were cultured and maintained in DMEM growth medium containing 10% FBS, as detailed previously (17). Cells were serum starved overnight in medium supplemented with 0.1% FBS before treatment with troglitazone (5 μM), pioglitazone (5 μM), or vehicle (DMSO) for 15 min.

Administration of troglitazone in vivo. Vehicle (DMSO-saline) and troglitazone (10 mg/kg) were injected intraperitoneally into control and experimental Sprague-Dawley rats, respectively. After 30 min, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), blood was collected, and gastrocnemius and soleus muscles, liver, and epididymal fat were quickly removed and quick-frozen in liquid N\textsubscript{2}. The tissues were stored at −80°C until they were analyzed.

Preparation of tissue and cell lysates. Tissues were homogenized on ice in buffer A [30 mM Na-HEPES, pH 7.4, 2.5 mM EGTA, 3 mM EDTA, 32% glycerol, 20 mM KCl, 40 mM β-glycerophosphate, 40 mM NaF, 4 mM NaPP\textsubscript{1}, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 0.1% Nonidet P-40, 2 mM diisopropyl fluorophosphate (DFP), 2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μM aprotinin, leupeptin, and pepstatin A, and 1 mM dithiothreitol (DTT)]. Homogenates were centrifuged (14,000 g for 10 min at 4°C) and supernatants were harvested, supplemented with an equal volume of buffer B [30 mM Na-HEPES, pH 7.4, 2.5 mM EGTA, 3 mM EDTA, 70 mM KCl, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM NaPP\textsubscript{1}, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 0.1% Nonidet P-40, 2 mM DFP, 2 mM PMSF, 5 μM aprotinin, leupeptin, and pepstatin A, and 1 mM DTT], and methods to harvest the supernatant were repeated. Cultured cells were scraped on ice in cell lysis buffer (plus 1 mM PMSF) and centrifuged (14,000 g for 15 min at 4°C). Protein concentrations of tissue and cell supernatants were determined by the Bradford method using dye reagent from Bio-Rad Laboratories, and bovine serum albumin served as the standard.

Immunoblot analysis. Aliquots of tissue (30 μg) and cell (25 μg) protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Membranes were blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 and 5% milk for 1 h at room temperature and then probed with antibodies specific to pan-AMPKα, pACC (Ser\textsuperscript{79}), or actin for 1 h at room temperature or specific to pAMPK (Thr\textsuperscript{172}) or PPAR\gamma overnight at 4°C. Bound antibodies were detected with the appropriate horseradish peroxidase-linked whole secondary antibodies. Protein immunoblots were visualized by enhanced chemiluminescence, and bands were quantified with scanning densitometry. The sizes of the antibody-bound proteins were verified using standard molecular mass markers.

Determination of AMPK activity. AMPKα2 activity was measured in EDL muscle, as described previously (43). In brief, lysate containing 200 μg of protein was immunoprecipitated with AMPKα2-specific antibody and protein A/G-agarose beads. Beads were washed, and the immobilized enzyme was assayed on the basis of phosphorylation of SAMS peptide (0.2 mmol/l) by 0.2 mmol/l ATP (containing 2 μCi [γ-\textsuperscript{32}P]ATP) in the presence and absence of 0.2 mmol/l AMP. Label incorporation into the SAMS peptide was measured using a scintillation counter.

Nucleotide studies. ATP, ADP, AMP, and phosphocreatine (PCr) were measured spectrophotometrically, as described previously (28, 33).

Analysis of adiponectin from rat serum. Following methods previously described (42), 2 μl of collected rat serum from vehicle- and troglitazone-treated animals were analyzed via SDS-PAGE/Western Blot strategy. Adiponectin was detected and quantified using an antisera directed against an epitope in its globular domain.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was undertaken using a paired Student’s t-test and one-way ANOVA. When ANOVA revealed significant differences, further analysis was performed using Tukey’s post hoc test for multiple comparisons. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Troglitazone activates AMPK in incubated skeletal muscle. Isolated rat EDL muscles incubated with troglitazone for 15 min demonstrated a significant increase in the phosphorylation of AMPK at Thr\textsuperscript{172}, indicating activation of the enzyme. The increase in phosphorylation was 1.9-fold at 5 μM troglitazone, with maximal phosphorylation occurring at 100 μM (7.4-fold; Fig. 1, A and B). Phosphorylation of the downstream target of AMPK, ACC (Ser\textsuperscript{79}), paralleled the increase in AMPK phosphorylation in a dose-dependent manner.

Time-course studies revealed that 5 μM troglitazone induced significant phosphorylation of AMPK and ACC after as little as 5 min of incubation, with peak increases occurring at 5–15 min. After this, phosphorylation of both AMPK and ACC decreased to basal levels by 60 min (Fig. 2, A and C). AMPKα2 subunit activity followed a similar pattern; however, activity was not significantly increased until 15 min (Fig. 2B). The disparity between these two measures (phosphorylation vs. activity) could be due to methodological differences, or it could possibly be due to allosteric modification of AMPK (i.e., activation by AMP and inhibition by PCr), independent of

E176 THIAZOLIDINEDIONES CAN RAPIDLY ACTIVATE AMPK

AJP-Endocrinol Metab • VOL 291 • JULY 2006 • www.ajpendo.org

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phosphorylation. Specifically, allosteric inhibition of AMPK may not be overcome by 5 μM troglitazone in as little as 5 min of incubation, despite phosphorylation at Thr172. Incubation with 100 μM troglitazone induced equally rapid, but more robust and prolonged, increases in the phosphorylation of AMPK and ACC and AMPK activity than did 5 μM troglitazone (Fig. 2, A–C). No alterations in total AMPK abundance were observed (data not shown).

Troglitazone acutely increases the AMP-to-ATP ratio. Troglitazone (100 μM) caused a rapid increase in the concentration of AMP and decreases in ATP and PCr that first appeared at 5 min and were maximal at 15 min. By 60 min the concentration of these high-energy compound phosphates had returned to baseline values like the abundance of pAMPK and pACC (Table 1).

Troglitazone stimulates glucose transport and fatty acid oxidation in skeletal muscle. To determine whether the activation of AMPK in skeletal muscle by troglitazone is associated with enhanced glucose transport, isolated EDL muscles were incubated in medium containing 2-DG in the absence or presence of troglitazone (100 μM), insulin (10 mU/ml), or both for 30 min. Relative to controls, troglitazone stimulated 2-DG transport 2.3-fold ($P < 0.02$) and insulin 3.6-fold. The effect of the two agents (4.0-fold) was not greater than that of insulin alone ($P = 0.51$; Fig. 3A). In keeping with its effects on AMPK
and ACC, troglitazone also increased fatty acid oxidation in the isolated EDL by 81% (Fig. 3B).

Troglitazone rapidly activates AMPK in vivo. Troglitazone also rapidly activated AMPK in rat tissues in vivo. Thirty minutes after the intraperitoneal administration of troglitazone (10 mg/kg), the phosphorylation of AMPK was increased (1.5- to 2.3-fold) in gastrocnemius and soleus muscles, liver, and epididymal fat (Fig. 4, A and B). Significant phosphorylation of ACC was observed in soleus, liver, and epididymal fat, and a similar trend was noted in the gastrocnemius (P = 0.09; Fig. 4, A and C). Plasma adiponectin levels tended to be lower (21.0 ± 11.0%) in troglitazone-treated rats (data not shown); however, the difference was not significant.

TZDs activate AMPK independently of PPARγ abundance. The rapidity of the effects of troglitazone on AMPK suggests they are not the result of PPARγ-mediated gene transcription.

Table 1. Troglitazone rapidly increases AMP and decreases ATP in skeletal muscle

<table>
<thead>
<tr>
<th>Incubation Time, min</th>
<th>Con</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>4.50±0.270</td>
<td>4.20±0.360</td>
<td>3.80±0.740</td>
<td>4.60±0.250</td>
<td>4.20±0.160</td>
</tr>
<tr>
<td>ADP</td>
<td>0.85±0.084</td>
<td>0.70±0.080</td>
<td>0.80±0.09</td>
<td>0.80±0.074</td>
<td>0.75±0.084</td>
</tr>
<tr>
<td>AMP</td>
<td>0.04±0.007</td>
<td>0.07±0.008*</td>
<td>0.10±0.020*</td>
<td>0.09±0.023*</td>
<td>0.04±0.004</td>
</tr>
<tr>
<td>CrP</td>
<td>13.5±1.06</td>
<td>10.00±0.910*</td>
<td>9.70±0.910*</td>
<td>11.7±0.950*</td>
<td>12.01±0.440</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7/group. CrP, creatine phosphate. Rat extensor digitorum longus muscles were incubated in the absence (Con) or presence of 100 μM troglitazone for indicated times. Muscle metabolite concentrations were determined by spectrophotometric assays and recorded in μM/g. *Significant differences compared with control values (P < 0.05).

To evaluate this question further, studies were performed in Swiss 3T3 fibroblasts expressing either control vector (3T3-Con) or retroviral PPARγ (3T3-PPARγ). It has been shown that treatment of the PPARγ-deficient 3T3-Con cells with 5 μM troglitazone does not increase the expression of FABP4/aP2, a well characterized target of activated PPARγ, whereas similar treatment of the 3T3-PPARγ cells, which contain eightfold more PPARγ, results in an expression of FABP4/aP2 (30). As shown in Fig. 5, incubation with 5 μM troglitazone for 15 min caused similar increases in the phosphorylation of...
TZDs can also acutely activate AMPK in mammalian tissue by activates AMPK. The results of the present study indicate that and an increase the level of adiponectin (27), an adipokine that 16, 30, 41, 47) that lead to diminished plasma levels of FFAs action by effects on gene transcription in the fat cell (7, 12, 15,

an effect independent of the fat cell and most likely PPARγ. Thus troglitazone activated AMPK in incubated rat EDL muscle within 15 min, an effect that was paralleled by a change in cellular energy state and an increase in both glucose uptake and fatty acid oxidation. In addition, the intraperitoneal administration of troglitazone acutely (30 min) activated AMPK signaling in rat liver and adipose tissue, indicating that this effect is not unique to skeletal muscle. Finally, troglitazone increased AMPK activation to the same extent in cultured fibroblasts in which endogenous PPARγ was insufficient to allow TZD-mediated gene expression as it did in cells in which PPARγ was overexpressed.

Rapid TZD-induced increases in AMPK have been reported in H-2Kb muscle cells treated with the TZD rosiglitazone (13) and L6 myotubes treated with 11 μM troglitazone (22). In the present study, the activation of AMPK in incubated skeletal muscle was accompanied by a decrease in the concentration of creatine phosphate and an increase in the AMP-to-ATP ratio, effects previously reported in H-2Kb and L6E9 myotubes (13, 22). It has been suggested that TZDs could cause such changes in adenine nucleotides by binding to and inhibiting mitochondrial proteins, including respiratory complex I (4, 5) and mitonene (9), or by otherwise reducing mitochondrial membrane potential (22). TZDs have also been reported to increase intracellular Ca\textsuperscript{2+} by causing its release from the endoplasmic reticulum (17); however, whether this leads to a change in energy state was not studied. Available evidence (4, 17) suggests that these effects of TZDs on mitochondrial proteins and membrane potential and cell calcium are not PPARγ mediated (4, 17).

Troglitazone increased AMPK activity within 5 min in incubated rat EDL muscles and within 30 min in multiple tissues in vivo when it was injected intraperitoneally. Both effects were too rapid to be attributable to PPARγ-mediated gene transcription. In keeping with this conclusion, acute activation of AMPK occurred in skeletal muscle, a tissue with minimal PPARγ, and it was of equal magnitude in fibroblasts with and without sufficient PPARγ to mediate the expression of target genes (26).

In the present study, activation of AMPK by troglitazone was associated with a >2-fold increase in 2-DG uptake in the incubated EDL at 30 min. A similar effect of troglitazone (10 μM) on glucose transport has been attributed to GLUT4 translocation in L6 myotubes incubated with the drug for 24 h (49). Furnsinn et al. (14) have reported that the stimulation of glucose transport by troglitazone (60 min incubation) in soleus

AMPK (2.0-fold) and ACC (1.6-fold) in 3T3-Con cells and 3T3-PPARγ cells (Fig. 5, B and C). Moreover, the effects of another TZD, pioglitazone (5 μM), on the two cells were indistinguishable from those of troglitazone (Fig. 6). Thus the acute activation of AMPK by TZDs in these cells does not appear to be dependent on PPARγ-mediated gene transcription.

**DISCUSSION**

Previous studies have suggested that TZDs improve insulin action by effects on gene transcription in the fat cell (7, 12, 15, 16, 30, 41, 47) that lead to diminished plasma levels of FFAs and an increase the level of adiponectin (27), an adipokine that activates AMPK. The results of the present study indicate that TZDs can also acutely activate AMPK in mammalian tissue by

![Fig. 5. TRO activates pAMPK independently of PPARγ expression in fibroblasts. Swiss-3T3 cells expressing either control vector (3T3-Con) or retroviral PPARγ (3T3-PPARγ) were treated with vehicle (Veh) or 5 μM TRO for 15 min. A: cell lysates were probed with antibodies specific for pAMPK, pACC, pan-AMPK-α, PPARγ and actin (n = 5). Summary of Western blot data (means ± SE) for pAMPK (A), pACC (B), and actin (C) for 3T3-PPAR γ treated with Veh (open bars) or 5 μM TRO (striped bars) (n = 5, *P < 0.05 vs. Veh).](image)

![Fig. 6. Pioglitazone (PIO) activates AMPK independently of PPARγ expression similar to TRO. Experiments conducted in 3T3 cells were repeated substituting the thiazolidinedione PIO (5 μM) for TRO. Representative blots for pAMPK, pACC, and actin (n = 4).](image)
muscle strips is associated with a decrease in oxidative metabolism (fatty acids) and noted that its effects were similar to those of hypoxia. In the present study, activation of AMPK by troglitazone in the incubated EDL was associated with a rapid inhibition of ACC (phosphorylation at Ser\(^{79}\)), which typically results in a decrease in the concentration of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase (CPT) I (29). In keeping with this, we observed an 80% increase in palmitate oxidation. Likewise, incubation with different TZDs (4 days of 11.5 \(\mu\)M troglitazone, 10 \(\mu\)M rosiglitazone, or 10 \(\mu\)M pioglitazone) has been shown to enhance depressed rates of palmitate oxidation and increase CPT I expression in muscle cells isolated from patients with type 2 diabetes (8). The basis for the differing effects of TZDs in previous reports and the present study and that of Fursinn remains to be determined.

As already noted, the effects of TZDs to diminish insulin resistance have been attributed to their ability to decrease plasma FFAs and alter the concentration of adiponectin and possibly other adipokines. The role of AMPK in mediating the action of TZDs has not been studied in humans; however, chronic treatment (2 wk) with either PIO (37) or rosiglitazone (48) increases AMPK activity in liver and adipose tissue of rats, and this closely correlates with the ability of these agents to diminish insulin resistance induced by raising plasma FFA levels during a euglycemic hyperinsulinemic clamp (48). Whether concurrent increases in plasma adiponectin contributed to the increase in tissue AMPK in these longer-term studies remains to be determined. In this context, it has recently been shown that the ability of 2 wk of rosiglitazone treatment (10 mg kg\(^{-1}\) day\(^{-1}\)) to activate AMPK in liver and muscle and enhance insulin action is diminished in adiponectin knockout mice (32).

In the present study, however, the rapid (30 min) activation of AMPK in vivo was not associated with an increase in the plasma concentration of immunosassayable adiponectin. The possibility that changes in adiponectin distribution (high molecular weight, trimmer, and hexamer) occur in vivo within 30 min of troglitazone injection was not examined, although to our knowledge, such rapid changes have not been described. It is also unlikely that adiponectin was a factor in the studies with incubated muscle and cultured cells, because adiponectin per se is produced only by adipose tissue. On the other hand, the existence of adiponectin-like molecules (paralogs) in multiple tissues (46) suggests that this possibility should not be ruled out.

It is important to note that the concentrations of troglitazone used in the present study are within the ranges reported to be biologically active in both rodents and humans. Using a cell-based PPAR\(\gamma\)-GAL4 transactivation assay, Willson et al. (45) have determined the EC\(_{50}\) of troglitazone and PIO to be in the range of 0.55–0.78 \(\mu\)M for both murine and human PPAR\(\gamma\). The concentrations of troglitazone used in vitro (5 \(\mu\)M) are within a log unit of the EC50s described in this report. In addition, Brown et al. (3) have reported that the in vivo antihyperglycemic efficacy (61% reduction in plasma glucose) of troglitazone occurred at an oral dose of 500 mg/kg in the Zucker diabetic fatty rat. This resulted in a plasma concentration of 44 \(\mu\)g/ml, or 99 \(\mu\)M. In the present study, the concentrations of troglitazone employed to characterize the rapid activation of AMPK were 10 mg/kg in vivo and 5–100 \(\mu\)M in isolated muscle.

In summary, troglitazone rapidly activates AMPK and increases the AMP-to-ATP ratio, glucose uptake, and fatty acid oxidation in mammalian skeletal muscle. Likewise in vivo, troglitazone activates AMPK signaling in muscle, liver, and adipose tissue within 30 min in the absence of an increase in adiponectin. It is generally held that TZDs increase insulin sensitivity and decrease inflammation by PPAR\(\gamma\)-mediated changes in the adipocyte that lead to a decrease in plasma FFAs and altered release of adiponectin and other adipokines. Whether the rapid activation of AMPK in mammalian tissue described here represents an additional mechanism by which TZDs exert their therapeutic action remains to be determined.

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