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

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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 independent of PPARγ-mediated gene transcription. Thus incubation of isolated rat EDL muscles in medium containing 5 μM 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-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^{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^{79}) antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Protein A/G sepharose beads, actin, PPARγ, 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-^3H]-glucose (2-DG), [U-^{14}C]mannitol, and [U-^{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_{2}-5% CO_{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 nU/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-^{14}C]mannitol (as an extracellular space marker) (23). Fatty acid oxidation was assessed on the basis of ^14CO_{2} release into the medium in muscle incubated with or without troglitazone (100 μM) and 0.2 mM 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 PPARγ overexpression.** Swiss-3T3 cells expressing either retroviral PPARγ (3T3-PPARγ) 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_{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 NaPPi, 1 mM Na_{2}VO_{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 diithiothreitol (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 NaPPi, 1 mM Na_{2}VO_{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^{79}), or actin for 1 h at room temperature or specific to pAMPKα (Thr^{172}) or PPARγ 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-^{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^{172}, indicating activation of the enzyme. The increase in phosphorylation was 1.9-fold at 5 μM troglitazone, with peak increases occurring at 100 μM (Fig. 2). Phosphorylation of AMPK, ACC (Ser^{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
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 Thr<sup>172</sup>. 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

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**Fig. 1.** Troglitazone (TRO) stimulates AMP-activated protein kinase (AMPK) phosphorylation in incubated skeletal muscle. Extensor digitorum longus (EDL) muscles were isolated and incubated in 0 to 250 μM TRO for 15 min. 

_A_: muscle lysates were then analyzed for phosphorylation of AMPK (pAMPK), acetyl-CoA carboxylase (pACC) and AMPKα using SDS-PAGE/ Western Blot strategy. 

_B_: density of pAMPK bands were quantified, and means were plotted ± SE (n = 4). *P* < 0.05 relative to 0 μM.

**Fig. 2.** TRO rapidly increases AMPK phosphorylation, activity, and signaling in incubated skeletal muscle. For time-course studies, EDL muscles were incubated in the absence (Con) or presence of 5 (left) or 100 μM (right) TRO for 0 to 60 min. Muscle lysates were analyzed for pAMPK (A), AMPK activity (B), and pACC (C) as described (MATERIALS AND METHODS), and results were summarized. Each bar represents the mean ± SE. *P* < 0.05 relative to controls (n = 7 muscles per time point at both 5 and 100 μM).
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.

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

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

<table>
<thead>
<tr>
<th>Incubation Time, min</th>
<th>ATP (µM)</th>
<th>ADP (µM)</th>
<th>AMP (µM)</th>
<th>ATP (µM/g)</th>
<th>ADP (µM/g)</th>
<th>AMP (µM/g)</th>
</tr>
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<tbody>
<tr>
<td>Con</td>
<td>4.50 ± 0.270</td>
<td>0.85 ± 0.084</td>
<td>0.04 ± 0.007</td>
<td>4.20 ± 0.360</td>
<td>0.70 ± 0.080</td>
<td>0.07 ± 0.008*</td>
</tr>
<tr>
<td>5</td>
<td>3.80 ± 0.740*</td>
<td>0.80 ± 0.09</td>
<td>0.10 ± 0.020*</td>
<td>3.80 ± 0.460</td>
<td>0.80 ± 0.074</td>
<td>0.09 ± 0.023*</td>
</tr>
<tr>
<td>15</td>
<td>4.60 ± 0.250</td>
<td>0.75 ± 0.084</td>
<td>0.04  ± 0.004*</td>
<td>4.60 ± 0.160</td>
<td>0.75 ± 0.084</td>
<td>0.04 ± 0.004</td>
</tr>
<tr>
<td>30</td>
<td>4.20 ± 0.160</td>
<td>0.75 ± 0.084</td>
<td>0.04 ± 0.004*</td>
<td>4.20 ± 0.160</td>
<td>0.75 ± 0.084</td>
<td>0.04 ± 0.004</td>
</tr>
<tr>
<td>60</td>
<td>4.20 ± 0.160</td>
<td>0.75 ± 0.084</td>
<td>0.04 ± 0.004*</td>
<td>4.20 ± 0.160</td>
<td>0.75 ± 0.084</td>
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Values are means ± SE; n = 7/group. Significant differences compared with control values (P < 0.05).

Fig. 3. TRO acutely stimulates glucose uptake and fatty acid oxidation in skeletal muscle. After isolation and preincubation, EDL muscles were incubated in the absence (Con) or presence of TRO (100 µM) 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).

Fig. 4. TRO acutely activates AMPK and ACC in vivo. Control and experimental rats were administered vehicle (DMSO/saline, –) and 10 mg TRO/kg (+), respectively. Thirty minutes after injection, animals were euthanized and tissues were removed. A: tissue lysates were analyzed using phosphospecific antibodies for AMPK (top), ACC (middle), and AMPKα (bottom). Quantification of densitometry data (means ± SE) for pAMPK (A) and pACC (C) for isolated tissues (n = 7 animals per group; *P < 0.05 vs. control, †P < 0.09).
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 in AMPK. The results of the present study indicate that 
TZDs can also acutely activate AMPK in mammalian tissue by 
an effect independent of the fat cell and most likely PPARγ. 
Thus troglitazone activated AMPK in incubated rat EDL mus- 
cle 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 adminis- 
tration 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 mitochon-
drial proteins, including respiratory complex I (4, 5) and 
mitoene (9), or by otherwise reducing mitochondrial mem-
bane potential (22). TZDs have also been reported to increase 
intracellular Ca2+ 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) sug-
gests 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 
icubated 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 
icubated 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

**Fig. 5.** TRO activates pAMPK independently of PPARγ expression in fibro-
blasts. 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 (C) or 3T3-Con and 3T3-PPARγ 
treated with Veh (open bars) or 5 μM TRO (striped bars) (n = 5, P < 0.05 vs. 
Veh).

**Fig. 6.** Pioglitazone (PIO) activates AMPK independently of PPARγ expres-
sion 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).
The concentrations of troglitazone used in vitro (5-29) range of 0.55–0.78 μM have determined the EC50s of troglitazone and PIO to be in the 44–98 μM range. In keeping with this, we observed an 80% increase in the activity of carnitine palmitoyltransferase (CPT) I in muscle and cultured cells, because adiponectin per se is unlikely that adiponectin was a factor in the studies with troglitazone in the incubated EDL was associated with a rapid 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 the activity of 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 Fursin 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 immunoassayable adiponectin. The possibility that changes in adiponectin distribution (high molecular weight, trimer, 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 PPARγ-GAL4 transactivation assay, Willson et al. (45) have determined the EC50s of troglitazone and PIO to be in the range of 0.55–0.78 μM for both murine and human PPARγ. The concentrations of troglitazone used in vitro (5 μM) 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 μg/ml or 99 μM. 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 μM 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 PPARγ-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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