Thiazolidinediones enhance skeletal muscle triacylglycerol synthesis while protecting against fatty acid-induced inflammation and insulin resistance

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1Department of Kinesiology and 2Department of Biological Sciences, University of Southern California, Los Angeles, California; 3St. Vincent’s Institute of Medical Research and the Department of Medicine, The University of Melbourne, Fitzroy, Australia; and 4Department of Medicine, University of California, San Diego, La Jolla, California

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Todd MK, Watt MJ, Le J, Hevener AL, Turcotte LP. Thiazolidinediones enhance skeletal muscle triacylglycerol synthesis while protecting against fatty acid-induced inflammation and insulin resistance. Am J Physiol Endocrinol Metab 292:E485–E493, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00080.2006.—In the present investigation, we studied the effects of thiazolidinedione (TZD) treatment on insulin-stimulated fatty acid (FA) and glucose kinetics in perfused muscle from high-fat (HF)-fed rats. We tested the hypothesis that TZDs prevent FA-induced insulin resistance by attenuating proinflammatory signaling independently of myocellular lipid levels. Male Wistar rats were assigned to one of three 3-wk dietary groups: control chow fed (CON), 65% HF diet (HFD), or TZD-(troglitazone or rosiglitazone) enriched HF diet (TZD+HFD). TZD treatment led to a significant increase in plasma membrane content of CD36 protein in muscle (red: P = 0.01, and white: P = 0.001) that correlated with increased FA uptake (45%, P = 0.002) and triacylglycerol (TG) synthesis (46%, P = 0.03) during the perfusion. Importantly, whereas HF feeding caused increased basal TG (P = 0.047), diacylglycerol (P = 0.002), and ceramide (P = 0.01) levels, TZD treatment only prevented the increase in muscle ceramide. In contrast, all of the muscle inflammatory markers altered by HF (P = 0.009; †IKKβ activity, P = 0.006; ‡IkB-α protein, P = 0.03; and †JNK phosphorylation, P = 0.003) were completely normalized by TZD treatment. Consistent with this, HFD-induced decrements in insulin action were also prevented by TZD treatment. Thus our findings support the notion that TZD treatment causes increased FA uptake and TG accumulation in skeletal muscle under insulin-stimulated conditions. Despite this, TZDs suppress the inflammatory response to dietary lipid overload, and it is this mechanism that correlates strongly with insulin sensitivity.

insulin action; lipid oversupply; nuclear factor-κB inflammatory pathway; lipotoxicity

INSULIN RESISTANCE, A HALLMARK FEATURE of type 2 diabetes and the metabolic syndrome, is characterized by impairments in long-chain fatty acid (FA) and glucose metabolism (6, 7, 19, 27, 36). Although elevated plasma FA and tissue lipids are often found clinically in humans with insulin resistance or type 2 diabetes (1, 19, 21, 36, 37) and are believed to be involved in the pathogenesis of skeletal muscle insulin resistance (1, 4, 19, 21, 27), the mechanisms by which chronically elevated lipids impact upon skeletal muscle metabolism remain poorly defined.

Recently, a causal link between lipid oversupply, inflammation, and impairments in insulin action has been proposed (3, 9, 16, 39). A role for proinflammatory serine kinases in the diminution of proximal insulin signal transduction is well supported (2, 3, 12, 15, 17, 24). The insulin receptor substrate (IRS)-1 has been targeted as a critical site for inflammatory impingement on insulin signaling since IRS-1 serine phosphorylation reduces activation of phosphatidylinositol (PI) 3-kinase and subsequent downstream signaling (2, 10, 22, 24). A host of serine kinases, including c-Jun NH2-terminal kinase (JNK), IkB kinase (IKKβ), and PKC0, have been identified as mediators of these deleterious events (2, 10, 12, 22, 24). Although the details and circumstances relating to specific kinase activation remain poorly described, accumulation of proinflammatory lipid intermediates, including ceramide, diacylglycerol (DAG), and long-chain fatty acyl CoAs, are believed to stimulate serine kinase activation, and it is thought that this mechanism underlies impaired insulin action (1, 8, 13, 17, 43).

In addition to impinging directly on insulin signal transduction, the activation of proinflammatory/stress signaling pathways, including nuclear factor (NF)-κB, alters insulin action by modulating gene transcription. Upon stimulation, IKKβ is phosphorylated, which leads to subsequent phosphorylation of the inhibitory subunit, IkBα, of the NF-κB complex (18). IkBα is then targeted for proteasomal degradation thus allowing for NF-κB translocation to the nucleus and transcriptional activation of proinflammatory target genes (e.g., TNF-α, IL-6, IL-1β) (18, 30, 54). Given its pivotal role in activating the NF-κB inflammatory pathway in regulating skeletal muscle insulin action (52, 53). Confirmation of a central role for this inflammatory pathway in regulating skeletal muscle insulin action was achieved in vitro in L6 myotubes whereby pharmacological inhibition of NF-κB nuclear translocation was shown to prevent FA-induced insulin resistance (41). Together, this work indicates that FA oversupply is associated with activation of the NF-κB inflammatory pathway and that reduced IKKβ activity or inhibition of NF-κB nuclear translocation prevents FA-induced decrements in insulin action (3, 41, 53).

To date, there is only modest in vivo data addressing the effect of clinically utilized insulin-sensitizing agents, such as

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TZDs, on skeletal muscle lipid metabolism and inflammation. Although it is believed by some (35, 40) that TZDs cause insulin sensitization in skeletal muscle via reductions in myocellular lipid levels, additional studies (14, 23, 26, 29) have shown that TZDs can prevent lipid-induced insulin resistance independently of a reduced lipid content. Given this, in the present investigation we sought to address the impact of TZD treatment on skeletal muscle lipid accumulation, inflammation, and insulin action following HF feeding. Here, we report that TZD treatment caused elevated rates of FA uptake and triacylglycerol (TG) synthesis in skeletal muscle from HF-fed rats perfused under insulin-stimulated conditions. Despite this, TZD treatment prevented the activation of IKKβ and JNK as well as the development of insulin resistance.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Wistar rats were randomly assigned to one of three 3-wk treatment groups distinguished by dietary composition. Animals assigned to the HF diet (HFD; n = 16) consumed 65% fat, 22% protein, and 13% carbohydrate (Dyets, Bethlehem, PA), whereas the TZD- (troglitazone or rosiglitazone) treated animals were fed the same HFD with the addition of troglitazone (TZD + HFD, 1.6%, n = 8) or rosiglitazone (TZD + HFD, 3 mg·kg⁻¹·day⁻¹, n = 8) as a food admixture. The control (CON) animals (n = 16) were fed normal chow containing 4% fat, 24% protein, and 72% carbohydrate (Harlan-Teklad, Madison, WI). All animals were given ad libitum access to food and water and were housed on a 12:12-h light-dark cycle. Animals were fasted for 8 h prior to basal blood or tissue sampling and prior to the start of the perfusion. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and were approved by the University of Southern California and the University of California, San Diego, Animal Subjects Committees.

**Perfusion**

Following the 8-h fast, blood from a tail vein was collected for baseline blood analysis. Hindquarters were surgically isolated. Quickly, the left superficial portion of the vastus lateralis muscle [white quadriceps (WQ)], the left superficial medial portion of the vastus lateralis [red gastrocnemius (RG)], and the deep medial portion of the vastus medialis muscle [white quadriceps (WQ)], the left superficial medial portion of the vastus medialis [red gastrocnemius (RG)], and the deep medial portion of the gastrocnemius muscle [red gastrocnemius (RG)] consisting mainly of fast-twitch glycolytic fibers, the deep portion of the vastus lateralis [red quadriceps (RQ)], and the deep medial portion of the gastrocnemius muscle [red gastrocnemius (RG)] consisting mainly of fast-twitch oxidative fibers were excised. Muscles were immediately visualized for fiber type distribution, sectioned, trimmed of connective tissue, blotted, and freeze-clamped with aluminum clamps precooled in liquid N₂ (45, 46, 48). Tissues were stored in liquid N₂ until subsequent analysis. The left iliac vessels were then tied off, and a clamp was fixed tightly around the proximal part of the leg to prevent bleeding.

Meanwhile, the right leg was perfused (7 ml/min) for an initial 20-min equilibration period followed by a 40-min experimental period with washed bovine erythrocytes containing [1-14C]palmitate (8 μCi), 600 μM palmitate, 6 mM glucose, and 100 μU/ml insulin as previously described by our laboratory (46–48). Arterial and venous perfusate samples for the analysis of [14C]FA and [14C]CO₂, as well as for FA and glucose concentrations, were taken at 10, 20, 30, and 40 min during the experimental period. Arterial and venous perfusate samples for determinations of Pco₂, Po₂, and pH were taken at 10 and 30 min. At the end of the 40-min experimental perfusion period, quadriceps (Q) and gastrocnemius (G) muscles of the right leg were immediately freeze-clamped in situ to preserve the insulin-induced activation state of the enzymes (45, 46, 48).

**Blood and Perfusate Analyses**

Plasma FA levels were measured enzymatically with a commercial kit (NEFA C; Wako Chemicals, Richmond, VA), glucose concentration was determined using the glucose oxidase method (YSI, Yellow Springs, OH), and Acrp30 and insulin were measured by radioimmunoassay kit (Linco, St. Louis, MO). Determination of Pco₂, Po₂, and pH of the perfusate samples was measured with an ABL5 analyzer (Radiometer America, Westlake, OH), and the collection of [14C]CO₂ liberated from the perfusate was performed as previously described (46–48). Per fusate concentration of [14C]palmitate was measured by liquid scintillation with a Hewlett-Packard scintillation counter (46–48).

**Skeletal Muscle Analyses**

Muscles were harvested and assessed under basal or insulin stimulated conditions. Analyses performed on basal muscle, including membrane fraction CD36 and PKC0 content (RG, 400 mg; WQ, 400 mg), were conducted on tissue harvested from the three groups of animals (CON, n = 8; HFD, n = 8; TZD + HFD, n = 8 troglitazone treated) prior to the perfusion. Insulin-stimulated analyses, including TG synthesis (mixed G, 400 mg), malonyl-CoA levels (mixed G, 200 mg), enzyme activities (mixed Q, 600 mg), and citrate levels (mixed Q, 100 mg), as well as insulin-signaling protein phosphorylation (mixed G, 250 mg), were conducted on tissues harvested from the three groups (CON, n = 8; HFD, n = 8; TZD + HFD, n = 8 troglitazone treated) following the perfusion. TG levels were assessed in basal muscle tissue from CON, n = 8; HFD, n = 8; and TZD + HFD, n = 8 troglitazone treated and n = 8 rosiglitazone treated, whereas lipid intermediates were assessed in basal muscle tissue from CON, n = 8; HFD, n = 8; and TZD + HFD, n = 8 rosiglitazone treated. IKKβ activity was assessed in insulin-stimulated mixed Q muscle (100 mg) harvested after the perfusion and correlated with the rate of insulin-stimulated glucose uptake during the perfusion. Assessment of inflammatory marker protein and phosphorylation content was performed in mixed muscle tissue (Q, 100 mg) harvested from each group (CON, n = 8; HFD, n = 8; TZD + HFD, n = 8 troglitazone treated) in the basal state. For simplicity, data from both TZD-treated groups (TZD + HFD, n = 8 troglitazone treated and n = 8 rosiglitazone treated) were collapsed for JNK phosphorylation since no differences were observed between the drug treatment groups.

**Enzymatic Activity Measurements**

Spectrophotometric analysis of β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was conducted to determine the potential for β-oxidation of FA (20). Briefly, mixed Q muscle was homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.2) containing 5 mM EDTA. The homogenate was centrifuged for 15 min, the supernatant was added to the reaction mixture (100 μM triethanolamine-HCl, 5 mM EDTA, 0.28 mM NADH, 0.25 mM acetoacetyl-CoA, pH 7.0), and absorption was followed at 340 nm for 5 min. Citrate synthase (CS) (42) activity was assayed for indication of TCA cycle flux using the mixed muscle supernatant described above. As previously described, acetyl-CoA carboxylase (ACC) activity was measured as the incorporation of [14C] from malonyl-CoA following ammonium sulfate precipitation of mixed Q muscle homogenates (34, 50). AMP-activated protein kinase (AMPK) activity was measured as the incorporation of [32P] from [32P]ATP into SAMS peptide following ammonium sulfate precipitation of mixed Q muscle homogenates (34, 50).

The IKK complex assay was performed following IKK complex immunoprecipitation of mixed Q muscle homogenate using an anti-IKKβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with protein A + G (1:1) sepharose beads (Upstate Biotechnology, Lake Placid, NY) as previously described (28). The beads were washed three times in 1) lysis buffer, 2) lysis buffer plus 2 M urea, and 3)
Table 1. Preperfusion characteristics of fasted animals in the basal state, parameters during the perfusion (100 μU/ml insulin), and postperfusion muscle characteristics

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>HFD</th>
<th>TZD + HFD</th>
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<tbody>
<tr>
<td>Preperfusion animal characteristics</td>
<td></td>
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<tr>
<td>Body weight, g</td>
<td>362±6.8</td>
<td>380±6.8</td>
<td>358±7.3</td>
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<tr>
<td>Plasma FA concentration, mmol/l</td>
<td>0.26±0.05</td>
<td>0.51±0.07*</td>
<td>0.26±0.05#</td>
</tr>
<tr>
<td>Plasma insulin concentration, ng/ml</td>
<td>0.47±0.05</td>
<td>0.73±0.08*</td>
<td>0.43±0.05#</td>
</tr>
<tr>
<td>Plasma Acrp30 concentration, μg/ml</td>
<td>15.4±1.5</td>
<td>11.0±0.9*</td>
<td>27.0±3.9*</td>
</tr>
<tr>
<td>Perfusion parameters</td>
<td></td>
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<tr>
<td>Oxygen uptake, μmol·g⁻¹·h⁻¹</td>
<td>20.8±1.7</td>
<td>20.5±1.5</td>
<td>18.9±1.5</td>
</tr>
<tr>
<td>Glucose concentration, mmol/l</td>
<td>9.5±0.3</td>
<td>9.9±0.4</td>
<td>9.9±0.2</td>
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<tr>
<td>FA concentration, mmol/l</td>
<td>0.61±0.02</td>
<td>0.65±0.02</td>
<td>0.62±0.02</td>
</tr>
<tr>
<td>FA delivery, mmol·min⁻¹·g⁻¹</td>
<td>103.3±1.9</td>
<td>105.9±3.2</td>
<td>105.6±3.9</td>
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<tr>
<td>Postperfusion muscle characteristics</td>
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<tr>
<td>Citrate concentration, mmol/g</td>
<td>187.9±12.1</td>
<td>336.9±13.6*</td>
<td>351.3±26.8*</td>
</tr>
<tr>
<td>Malonyl-CoA concentration, mmol/g</td>
<td>0.48±0.07</td>
<td>0.35±0.07</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>β-HAD activity, μmol·g⁻¹·min⁻¹</td>
<td>5.3±0.5</td>
<td>8.8±0.9*</td>
<td>8.0±0.4*</td>
</tr>
<tr>
<td>CS activity, μmol·g⁻¹·min⁻¹</td>
<td>16.5±0.61</td>
<td>19.2±0.6*</td>
<td>17.9±0.6</td>
</tr>
<tr>
<td>AMPK activity, mmol·mg⁻¹·min⁻¹</td>
<td>0.276±0.042</td>
<td>0.291±0.19</td>
<td>0.253±0.015</td>
</tr>
<tr>
<td>Vₐₘₜ for citrate activation of ACC, mmol·g⁻¹·min⁻¹</td>
<td>22.3±1.6</td>
<td>22.9±1.0</td>
<td>23.5±1.1</td>
</tr>
<tr>
<td>Kₐₜ for citrate activation of ACC, mM</td>
<td>5.6±0.9</td>
<td>4.9±0.7</td>
<td>4.4±0.7</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for the 3 groups. CON, control; HFD, high-fat diet; TZD, thiazolidinedione; FA, fatty acid; β-HAD, β-hydroxacyl-CoA dehydrogenase; CS, citrate synthase. AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase. Basal preperfusion measurements were made on control (CON) normal-chow diet (n = 16), HFD (n = 16), and TZD + HFD (n = 8 and n = 8, rosiglitazone- and troglitazone-treated, respectively) rats. Data from rosiglitazone- and troglitazone-treated rats were collapsed for body weight, plasma FA, insulin, and Acrp30 concentrations, since there were no significant differences between these 2 groups. Perfusion parameters and postperfusion muscle characteristics were measured on CON (n = 8), HFD (n = 8), and TZD + HFD (n = 8, troglitazone-treated) rats. *Significant difference vs. CON (P<0.05); #significant difference, HFD vs. TZD (P<0.05).

**Muscle Metabolite Measurements**

Malonyl-CoA levels were determined using neutralized perchloric acid extracts prepared from freeze-clamped mixed G muscle sampled after the perfusion as previously described (34). To measure the incorporation of [14C]palmitate into muscle TG, lipids from postperfusion muscle were extracted and separated and lipids from the extracted organic layer were separated by liquid chromatography as previously described (45).

Total citrate concentration was determined spectrophotometrically at 340 nm (33). Briefly, postperfusion Q muscle was homogenized 1:2 in 0.6 N perchloric acid and centrifuged for 5 min. The supernatant was neutralized with 2 N potassium hydroxide and incubated on ice for 15 min. The sample was centrifuged again, and the supernatant was added to the reaction mixture containing 25 mM Tris·HCl, pH 7.6, 0.2 mM NADH, 0.04 mM MnZnSO₄, and 0.3 U/ml malate dehydrogenase (Roche, Indianapolis, IN). Citrate concentration was calculated after the addition of 0.12 U/ml citrate lyase (Roche).

To measure muscle TG, DAG, and ceramide contents, lipids were extracted by the Folch method from basal Q muscle dissected free of visible connective tissue and blood (23). TG was saponified in an ethanol-hydroxide solution at 60°C, and glycerol content was determined fluorometrically. DAG and ceramide were extracted and quantified as previously described (23). Lipids were extracted from freeze-dried powdered muscle using chloroform-methanol-PBS + 0.2% SDS (1: 1/8). DAG kinase and [33P]ATP (15 μCi/mmol cold ATP) were added to lysates preincubated with cardiopin/oyctylglycoside, and the reaction was stopped after 2 h by the addition of chloroform-methanol (2:1). Samples were spotted onto TLC plates and developed. P-labeled phosphatic acid and ceramide 1-phosphate ceramide were identified, dried, scraped from the TLC plate, and counted in a liquid scintillation counter (Tri-Carb 2500TR; Packard, Canberra, Australia).

**Western Blot Analysis**

Plasma membrane fractions from preperfusion RG and WG were isolated from continuous sucrose gradient centrifugation for determination of CD36 transporter protein expression and translocation of PKC0 (Santa Cruz Biotechnology) as previously described (46). For the measurement of total content and/or activation state of the insulin transduction and inflammatory pathways, frozen samples from pre- and postperfusion muscle were homogenized in liquid N₂ and treated with lysis buffer containing phosphatase and protease inhibitors. After a 10-min incubation, lysates were clarified by centrifugation (10,000 g at 4°C) and supernatants analyzed for total protein content or phosphorylation state (Bio-Rad, Hercules, CA). Proteins were separated by SDS-PAGE on 7.5, 10, or 12% resolving polyacrylamide gels. Equal amounts of protein from each muscle sample were loaded onto each gel.

Followed electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) and incubated with the following antibodies according to the manufacturer’s instructions: anti-CD36 mouse monoclonal antibody (Cascad BioSciences, Winchester, MA), anti-α-actin (Sigma, St. Louis, MO), anti-IRS-1 (Upstate Biotechnology), mouse monoclonal IRS-1 anti-phosphotyrosine antibody (PY-20; Transduction Laboratories, Lexington, KY), IRS-1 anti-phosphoserine 307 antibody (Cell Signaling), anti-Akt1/2 antibody (Cell Signaling), anti-NIK (Santa Cruz), anti-IKKβ antibody (Cell Signaling), anti-IκBα antibody (Cell Signaling), anti-
Compared with CON, 3 wk of HF feeding led to a significant increase in circulating levels of TG, FA, and ceramide (Fig. 1, A, B, and C, respectively). The addition of a TZD to the diet significantly increased muscle ceramide content compared with HFD alone. However, the TZD-enriched diet did cause a significant reduction in muscle ceramide content to a level below that measured for HFD and CON (P = 0.0013; Fig. 1C).

Hindlimb Perfusion Parameters

Palmitate kinetic parameters in blood and muscle were measured as previously described (46–47). Because there were no significant differences in values measured after 20, 30, and 40 min of perfusion, average values were used for each animal. Palmitate oxidation was corrected for label fixation using a previously determined acetate correction factor of 1.6 for all groups as previously described (48). Statistical significance was determined using ANOVA with Newman-Keuls test for post hoc comparisons where appropriate, with the level of significance set a priori at P < 0.05.

RESULTS

Baseline Circulating Blood Parameters and Muscle Lipids

There were no detectable differences in animal body weights between the groups prior to or following the dietary intervention with or without the addition of TZD (P = 0.1, Table 1). Compared with CON, 3 wk of HF feeding led to a significant increase in both fasting plasma insulin (55% increase, P = 0.023) and FA levels (96%, P = 0.014), both of which were normalized by TZD treatment (P = 0.49 and P = 0.99 vs. CON, respectively; Table 1). Fasting plasma Acrp30 levels were significantly reduced by HF feeding (29% decrease, P = 0.026) but were significantly elevated above CON (75%, P = 0.003) following TZD treatment.

In a subset of animals not participating in perfusion experiments, Q muscle was harvested in the basal state and analyzed for lipid content. Three weeks of HF feeding led to a significant increase in content of muscle TG (86%, P = 0.047), DAG (45%, P = 0.002), and ceramide (70%, P = 0.01) compared with CON rats (Fig. 1, A, B, and C, respectively). The addition of a TZD to the HFD did not reduce muscle TG (P = 0.36) or DAG (P = 0.32) content compared with HFD alone. However, the TZD-enriched diet did cause a significant reduction in muscle ceramide content to a level below that measured for HFD and CON (P = 0.0013; Fig. 1C).

Palmitate metabolism. The protein level for the FA transporter CD36 was unchanged following HF feeding; however, TZD treatment caused a significant increase in CD36 protein expression in plasma membrane fractions isolated from RG muscle (Fig. 2A). Consistent with this, whereas the total uptake of palmitate was not affected by 3 wk of HF feeding, the addition of a TZD to the diet significantly increased muscle palmitate uptake by 45% (P = 0.002) during perfusion with insulin (Fig. 2B).

Compared with CON, palmitate oxidation, when expressed as total oxidation or a percentage of palmitate uptake, was increased in both HFD (65%, P = 0.002; and 63%, P = 0.03, respectively) and TZD + HFD (53%, P = 0.0007; and 105%, P = 0.001, respectively; Fig. 2C). Palmitate oxidation averaged 12.0 ± 0.8% of the total palmitate uptake in HFD and HFD + TZD and was not different between these two groups. Consistent with the role of malonyl-CoA in the regulation of FA oxidation, postperfusion malonyl-CoA concentration was negatively correlated with FA oxidation (R² = −0.42, P = 0.008; data not shown). Whereas malonyl-CoA levels were not different between the three groups, citrate levels increased...
almost identically by ~80% for HFD and TZD + HFD (P = 0.0001; Table 1) vs. CON.

To assess whether HFD-induced changes in key oxidative enzymes in muscle perfused under insulin-stimulated conditions, we measured the activities of β-HAD and CS, marker enzymes for the pathways of β-oxidation and the Krebs cycle, respectively. We observed a significant (51–64%) increase in β-HAD activity for HFD (P < 0.004) and TZD + HFD (P < 0.03; Table 1) compared with CON. CS activity was elevated only in HFD animals (P = 0.008) compared with CON. AMPK and ACC activities, shown to be important in the regulation of FA oxidation in skeletal muscle, were also assessed. However, no differences in AMPK and ACC activity or the K_m for citrate activation of ACC were detected between the groups (Table 1) during insulin stimulation.

Despite elevated lipid levels in muscle prior to perfusion, we observed a significant increase in palmitate uptake in TZD + HFD muscle, and this was associated with a 57 and 46% increase in the incorporation of [14C]palmitate into muscle TG relative to CON (P = 0.014) and HFD (P = 0.03) alone (Fig. 2D). Collectively, these data indicate that TZD treatment during HF feeding leads to an upregulation of FA transport capacity, translating into greater FA uptake as well as oxidative and nonoxidative metabolism.

Glucose uptake and insulin action. HF feeding was associated with a 47% (P < 0.001) reduction in skeletal muscle insulin-stimulated glucose uptake during the perfusion (Fig. 3A). In contrast, muscle glucose uptake in the HF-fed animals treated with TZD was not different from that measured in the control group (P = 0.1).

To assess the impact of HF diet and TZD treatment on skeletal muscle insulin signaling, muscle protein content and phosphorylation levels were analyzed using Western analysis on crude homogenates of the hindlimb. Total IRS-1 protein...
Akt serine (Ser473) phosphorylation was significantly increased (P = 0.01; Fig. 3A) following HF feeding. In agreement with this, IKKβ activity was also increased approximately twofold (P = 0.006; Fig. 4B) in muscle from HF-fed animals. The activation of IKKβ was significantly correlated with insulin-stimulated glucose uptake (R² = -0.67, P = 0.005; Fig. 4C).

Conventionally, IKK phosphorylates the inhibitory subunit of the NF-κB complex, IkBα, targeting this inhibitory molecule for rapid ubiquitin-proteasome degradation (18, 28, 52, 54). The release of this inhibitory subunit (IkBα) allows for NF-κB nuclear translocation and modulation of target gene transcription. Consistent with this paradigm, the protein content of IkBα was diminished by ∼25% (P = 0.03; Fig. 4A) in HFD muscle. HF feeding also increased NF-κB p65 total protein by 30% vs. CON (P = 0.05; Fig. 4A), and this increase was prevented by TZD treatment. Whereas HF feeding led to a marked increase in the protein level and activity of key NF-κB signaling molecules, the addition of TZD to the HFD completely suppressed FA-induced activation of this pathway.

Additionally, it is reported that other serine kinases are activated by HF feeding (15, 40). PKCδ is thought to be involved in FA-induced insulin resistance by phosphorylating IRS-1 at serine 1101 (24). Following HF feeding and muscle perfusion, we were unable to detect activation of PKCδ, as reflected by identical protein content levels in the total homogenate, plasma membrane, and particulate fractions between the three groups (P = 0.26; data not shown). This observation is consistent with that of Lessard et al. (23).

In contrast with observations made for PKCδ, HF feeding was associated with a 1.7-fold increase (P = 0.003; Fig. 4D) in JNK phosphorylation, a molecule also known to serine phosphorylate IRS-1 (15, 22). HFD-induced phosphorylation of JNK was completely reversed by dietary TZD enrichment. Total JNK protein levels were not different between the groups (P = 0.11; Fig. 4D).

**DISCUSSION**

Several studies have tested whether TZD treatment improves insulin action by reducing TG and lipid metabolites in muscle. However, to date, the findings are equivocal (21, 23, 26, 29, 35). Studies employing 3H nuclear magnetic resonance spectroscopy (26) or quantitative biochemical lipid analyses (23, 29) show that TZD-induced insulin sensitization in muscle occurs independently of changes in muscle lipid content. Due to these inconsistencies in the literature, we sought to test an alternative hypothesis that TZDs maintain insulin action during lipid oversupply by suppressing inflammation.

Our data confirm that HF feeding leads to the accumulation of lipid and lipid intermediates (including DAG, ceramide, and TG) in skeletal muscle, and this is associated with increased activation of specific serine kinases (IKKβ and JNK) and reduced insulin action. Consistent with our hypothesis, skeletal muscle insulin sensitivity is maintained in rats fed a HF diet enriched with a TZD, and this is accompanied by the suppression of proinflammatory signaling and reduced muscle ceramide content. TZD-induced protection of insulin action in skeletal muscle markers of inflammation

To address the lipotoxic effects of HF feeding on the stress-activated NF-κB inflammatory pathway, we measured expression was not different between the groups (P = 0.36; data not shown). The HF diet caused a fourfold (P = 0.001; Fig. 3B) elevation in IRS-1 serine phosphorylation and was associated with impaired IRS-1 tyrosine phosphorylation (Δ45%, P = 0.001; Fig. 3B) compared with CON. The addition of a TZD to the HF diet prevented serine phosphorylation and restored tyrosine phosphorylation of IRS-1 to CON levels.

Downstream of IRS-1 and PI 3-kinase, Akt protein levels were found to be identical between groups (P = 0.58; data not shown). Akt serine (Ser473) phosphorylation was significantly reduced (35%, P = 0.01; Fig. 3B) by HF feeding, and, consistent with the upstream signaling events, TZD enrichment of the HF diet restored insulin-stimulated Akt phosphorylation to CON levels.

**Skeletal Muscle Markers of Inflammation**

To address the lipotoxic effects of HF feeding on the stress-activated NF-κB inflammatory pathway, we measured
skeletal muscle occurred despite elevated rates of FA uptake and TG synthesis that resulted in increased TG accumulation. Thus these data contrast with reports suggesting that TZDs enhance insulin action by reducing FA storage in skeletal muscle (35, 40). To the contrary, we provide novel findings showing that the maintenance of insulin action occurs at least in part via suppression of proinflammatory signaling that is not associated with reduced lipid transport capacity or lipid accumulation.

Elevated circulating FA and TG, as well as excessive lipid storage within insulin-sensitive tissues, are associated with insulin resistance and are often found in type 2 diabetic patients or during experimental lipid oversupply (4, 7, 19, 32, 37). Although there is compelling evidence to suggest that excess cellular lipids play an etiological role in the development of insulin resistance in skeletal muscle (13, 17, 32), it is also known, but sometimes overlooked, that, under certain metabolic conditions (e.g., chronic exercise), high cellular lipids may be present without the presence of insulin resistance (5, 11, 38). This suggests that there must be some other cellular event that mediates FA-induced insulin resistance. In the present investigation, we sought to address this question (i.e., the lipid paradox) in TZD-treated HF-fed rats and investigate the role of the NF-κB proinflammatory signaling cascade as a direct mediator of FA-induced insulin resistance in skeletal muscle.

The notion that TZDs cause insulin sensitization due to reductions in skeletal muscle lipid accumulation would suggest that TZDs diminish FA uptake by muscle and/or elevate fatty acid oxidation so that less lipid is stored in muscle. If the primary mechanism for TZD-induced skeletal muscle insulin sensitization is enhanced FA partitioning into adipose tissue, thus sparing skeletal muscle form the lipotoxic effects of FA oversupply, a downregulation of skeletal muscle FA transport capacity should logically follow. Consistent with the published findings by several other groups (14, 31, 49), here we report that FA transport capacity, as measured by plasma membrane CD36 protein content, is increased in both red and white muscle following 3 wk of TZD treatment. Because plasma membrane CD36 was also measured following insulin stimu-
lotion, our results suggest that, compared with HFD alone, TZD treatment either increased total CD36 protein expression, restored muscle’s sensitivity to the effects of insulin on CD36 translocation (25), or both. The TZD-induced increase in plasma membrane CD36 content was associated with a 45% increase in FA uptake into skeletal muscle, and this was paralleled by a 43% increase in TG synthesis. These data indicate that the additional FA taken up during the perfusion by muscle from TZD-treated HF-fed rats vs. HFD alone were partitioned to storage. These data parallel recent work showing increased FA transport capacity, uptake, and/or TG accumulation in muscle following TZD treatment in rodents and obese or type 2 diabetic humans (23, 26, 29, 49).

Concurrent with these events, our findings show that TZDs suppress the proinflammatory response to FA oversupply, and this suppression presumably allows for normal insulin signaling to proceed. It is well known that the production of lipid intermediates, including DAG and ceramide, is associated with the activation of serine kinases (i.e., JNK and IKKβ) shown to diminish proximal insulin signaling in vitro (2, 10, 43). In vivo, systemic or tissue specific knockouts of these kinases have confirmed their role in mediating diet-induced insulin resistance (3, 15, 53). Here we report that HF feeding causes increased muscle accumulation of ceramide and DAG, which is paralleled by increased NIK expression, IKKβ activation, and JNK phosphorylation. Although the exact circumstances leading to serine kinase activation are unknown, both IKKβ and JNK have been shown to impair proximal insulin signaling via serine phosphorylation of IRS-1, a finding confirmed in the present investigation (22, 41). We also report that TZD treatment during HF feeding diminishes NIK protein expression and reduces the activation states of IKKβ and JNK. Our findings for TZD-treated HF-fed animals are consistent with those of the serine kinase knockout mice, where functional or pharmacological inactivation of IKKβ or JNK prevents HF diet-induced insulin resistance (3, 15, 53).

In conclusion, there is strong evidence that chronic inflammation is a major cause of insulin resistance. In the present investigation, we demonstrate that TZD treatment during HF feeding promotes insulin-stimulated skeletal muscle FA uptake and TG synthesis without activation of IKKβ and JNK or impairments in insulin action. We propose the notion that TZD treatment in the presence of dietary lipid overload protects muscle from FA-induced insulin resistance by suppression of inflammation that is independent of myocellular lipid levels.

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