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

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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). 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 (FA) (\( P = 0.002 \)), 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 feeding (\( \uparrow \) NIK protein content, \( P = 0.009 \); \( \uparrow \) IKK\( \beta \) activity, \( P = 0.006 \); \( \downarrow \) IkB-\( \alpha \) protein, \( P = 0.03 \); and \( \downarrow \) 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-\( \kappa \)B 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.

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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 (IKK\( \beta \)), and PKC\( \varepsilon \), 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)-\( \kappa \)B, alters insulin action by modulating gene transcription. Upon stimulation, IKK\( \beta \) is phosphorylated, which leads to subsequent phosphorylation of the inhibitory subunit, IkB\( \varepsilon \), of the NF-\( \kappa \)B complex (18). IkB\( \varepsilon \) is then targeted for proteasomal degradation thus allowing for NF-\( \kappa \)B translocation to the nucleus and transcriptional activation of proinflammatory target genes (e.g., TNF-\( \alpha \), IL-6, IL-1\( \beta \)) (18, 30, 54). Given its pivotal role in activating the NF-\( \kappa \)B inflammatory cascade, IKK\( \beta \) has been targeted clinically for pharmacological intervention as well as experimentally by genetic manipulation in mice. It is well known that salicylates ameliorate diabetic complications (16), and these clinical findings are supported experimentally in that similar treatment of high-fat (HF)-fed or genetically obese rodents causes reductions in IKK\( \beta \) activity and improved insulin action (52, 53). Moreover, mice heterozygous for the null IKK\( \beta \) mutation as well as IKK\( \beta \) tissue-specific knockouts are also protected from insulin resistance induced by HF feeding or leptin deficiency (3, 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-\( \kappa \)B 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-\( \kappa \)B inflammatory pathway and that reduced IKK\( \beta \) activity or inhibition of NF-\( \kappa \)B 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
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 myo-cellular 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 gastrocnemius muscle [white gastrocnemius (WG)] 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 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 anABL5 analyzer (Radiometer America, Westlake, OH), and the collection of 14CO₂ liberated from the perfusate was performed as previously described (46–48). Perfusate 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 PKCε content (RG, 400 mg; WG, 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 Q, 400 mg), malonyl-CoA levels (mixed Q, 200 mg), enzyme activities (mixed Q, 600 mg), and citrate levels (mixed Q, 100 mg), as well as insulin-signaling protein phosphorylation (mixed Q, 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 = 4 troglitazone treated and n = 6 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 mM 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 [1-14C]acetate into 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 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)
kinase buffer (20 mM HEPES, pH 7.5, 10 MgCl₂). The beads were harvested, and the kinase assay was performed for an incubation period of 30 min at 30°C using 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate 1 mM dithiothreitol, 50 μM sodium vanadate, 20 μM cold ATP, 0.5 μM [³²P]ATP, and 2 μg glutathione-S-transferase-IκBα (1–317, Santa Cruz-4094). The reaction was terminated by adding SDS-PAGE loading buffer and by heating the samples to a boiling temperature. The samples were then run on a 10% polyacrylamide gel, transferred to nitrocellulose membranes, and exposed to X-ray film. Band intensities were quantified by densitometry on a Hewlett-Packard ScanJet II using NIH Image 1.6 software. To guarantee equal protein loading per lane, the nitrocellulose membranes were blotted with an anti-IKKβ antibody (Santa Cruz Biotechnology), and total protein was expressed in arbitrary densitometry units on the basis of internal loading buffer and by heating the samples to a boiling temperature.

Muscle Metabolite Measurements

Malonyl-CoA levels were determined using neutralized perchloric acid extracts prepared from freeze-clamped mixed G muscle sample after the perfusion as previously described (34). To measure the incorporation of [¹⁴C]palmitate into muscle TG, lipids from postperfusion mixed G 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 supernant 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-HCl solution at 60°C, and glycerol content was determined fluorometrically. DAG and ceramide were extracted and quantified as previously described (25). Lipids were extracted from freeze-dried powdered muscle using chloroform-methanol-PBS + 0.2% SDS (1:2:2). DAG kinase and [³²P]ATP (150 μCi/mmol cold ATP) were added to lysates preincubated with cardiolipin/octylglucoside, and the reaction was stopped after 2 h by the addition of chloroform-methanol (2:1). Samples were spotted onto TLC plates and developed. ³²P-labeled phosphatidic 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 PKCθ (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 protein 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.

Following 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 and [³²P]ATP (15 μCi/mmol cold ATP) were added to lysates preincubated with cardiolipin/octylglucoside, and the reaction was stopped after 2 h by the addition of chloroform-methanol (2:1). Samples were spotted onto TLC plates and developed. ³²P-labeled phosphatidic 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).

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JNK antibody (Cell Signaling), anti-phospho-JNK antibody (Thr\(^{183}\)/Tyr\(^{185}\); Cell Signaling), and anti-NF-κB antibody (p65; Santa Cruz Biotechnology). Protein content was normalized to α-actin (found to be identical between the 3 groups, \(P = 0.67\)), and phospho-blots were normalized to the content of each respective protein. All phospho-blots were stripped and reprobed for protein content to confirm molecular weight. Band intensities were quantified by densitometry on a Hewlett-Packard ScanJet II using NIH Image 1.6 software. Figures generated from Western analyses contain representative blots and graphs of mean values ± SE expressed in arbitrary units obtained from densitometric analysis.

Calculations and Statistical Analyses

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. 1A, 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).

Hindlimb Perfusion Parameters

Perfusate FA (\(P = 0.39\)) and glucose (\(P = 0.41\)) concentrations and FA delivery to the muscle (\(P = 0.87\)), as well as muscle oxygen uptake (\(P = 0.67\)), were not different between the groups (Table 1).

Transport Capacity and Substrate Metabolism

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 (60% increase, \(P = 0.01\)) and WG (100% increase, \(P = 0.001\)) 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 (\(R^2 = -0.42, P = 0.008\); data not shown). Whereas malonyl-CoA levels were not different between the three groups, citrate levels increased
almost identically by \sim 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 HAD and CS, marker enzymes for the pathways of \( \beta \)-oxidation and the Krebs cycle, respectively. We observed a significant (51–64\%) increase in 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 [\( ^{14} \)C]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...
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 \((\downarrow 45\%; P = 0.001;\) 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 P3-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-kB inflammatory pathway, we measured the relative protein expression, the phosphorylation states, and the activity level of key signaling molecules within this pathway. NF-kB-inducing kinase (IKK), the purported upstream kinase involved in the activation of the IKK complex \((44),\) was shown to be elevated almost twofold \((P = 0.009;\) Fig. 4A) 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 \(\left(R^2 = -0.67, P = 0.005;\right)\) Fig. 4C).

Conventionally, IKK phosphorylates the inhibitory subunit of the NF-kB complex, IκBα, targeting this inhibitory molecule for rapid ubiquitin-proteasome degradation \((18, 28, 52, 54).\) The release of this inhibitory subunit (IκBα) allows for NF-kB nuclear translocation and modulation of target gene transcription. Consistent with this paradigm, the protein content of IκBα was diminished by \(\sim 25\% \left(P = 0.03;\right)\) Fig. 4A) in HFD muscle. HF feeding also increased NF-kB 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-kB 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 \((15, 40).\) PKCα 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 PKCα, 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 PKCα, 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 \(^{1}H\) 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 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 from 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-
loration, 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 muscle from FA-induced insulin resistance by suppression of 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 suppression of lipid metabolism and insulin resistance in rat and human muscle. 

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