We sought to ascertain whether pretreatment with troglitazone (20 days) could prevent acute free fatty acid (FFA)-induced insulin resistance in male Wistar rats. Animals were divided into three groups: 1) control, 2) FFA infusion alone (FFA1), and 3) thiazolidinedione (TZD)-treated + FFA infusion (FFA1). Days before a hyperinsulinemic-euglycemic clamp, all animals were cannulated in the jugular vein (infusion) and carotid artery (sampling). Animals were allowed 5 days to recover from surgery and fasted 12 h before the experiment. Glucose (variable), insulin (40 μU·kg⁻¹·min⁻¹), and Liposyn (heparinized 10% lipid emulsion) infusions were initiated simultaneously and continued from 0–120 min. Steady-state glucose, 8.3 ± 0.14 mmol/l, and insulin concentrations, 7.3 ± 2.45 mmol/l, were the same between groups. Interestingly, steady-state FFA levels were significantly lower in animals pretreated with TZD compared with FFA alone (1.83 ± 0.26 vs. 2.96 ± 0.25 mmol/l; P = 0.009), despite matched intralipid infusion rates. A second group of TZD-treated animals (TZD + FFA2) were infused with intralipid at a higher infusion rate (44%) to match the arterial concentrations of FFA1. The glucose infusion and insulin-stimulated glucose disposal rates (GDRs) were significantly decreased (40%) for untreated Liposyn infused (FFA1) compared with control rats. In addition, insulin receptor substrate-1 (IRS-1) phosphorylation and IRS-1–associated phosphatidylinositol (PI) 3-kinase activity was significantly reduced, 30–50%, in FFA1 rats. TZD pretreatment prevented the FFA-induced decrement in insulin signaling. Fatty acid translocase (FAT/CD36) total protein was significantly reduced (56%) in untreated FFA1 rats after the clamp but remained identical to control values for TZD-treated rats. In conclusion, acutely elevated FFA levels 1) induced a significant reduction in tracer-determined GDR paralleled by impaired tyrosine phosphorylation of IRS-1 and reduced IRS-1–associated PI 3-kinase activity and 2) induced a significant reduction in FAT/CD36 total protein. TZD pretreatment prevented FFA-induced decrements in insulin action and prevented the reduction in FAT/CD36 protein. *Diabetes* 50: 2316–2322, 2001

It is widely known that experimental elevation of circulating free fatty acid (FFA) levels leads to insulin resistance in animals and humans (1–3). This may represent a physiologic mechanism of insulin resistance, because elevated FFA levels are generally observed in most human insulin-resistant states (4,5). Thus, it is possible that chronic elevation of endogenous FFAs contributes to the insulin resistance in many pathophysiologic conditions. The mechanisms underlying FFA-induced insulin resistance are unclear, but recent evidence suggests that this lipotoxic effect of FFAs leads to decreased insulin-stimulated skeletal muscle glucose transport, and it is possible that this is due to some impairment in insulin signaling (6,7).

Thiazolidinediones (TZDs) are insulin-sensitizing agents that work by binding to peroxisome proliferator–activated receptor-γ (PPAR-γ) nuclear receptors, which leads to alteration in the expression of key glucoregulatory genes (8,9). It is known that TZDs are effective in a variety of insulin-resistant states and can protect animals and cells against insulin resistance induced by perturbations such as high fructose feeding and acute tumor necrosis factor-α infusion (10–13). In many settings, TZDs also lead to a reduction in FFA concentrations, and it has been suggested that this is an important mechanism underlying the TZD-induced insulin sensitization (14,15). In other words, TZDs may have a primary effect on adipose tissue to reducing circulating FFA levels, and this, in turn, leads to skeletal muscle insulin sensitization.

In the current study, we used a triglyceride emulsion infusion in combination with hyperinsulinemic-euglycemic clamps to assess the impact of elevated FFA levels on overall insulin action. Troglitazone, a well-studied TZD, is a partial agonist for the PPAR-γ receptor (16). Animals were pretreated with or without troglitazone to determine whether this TZD could protect against FFA-induced insulin resistance. Second, to assess whether troglitazone can improve skeletal muscle insulin sensitivity independent of a reduction in FFA concentration, we clamped the arterial FFA concentration at identical levels between TZD and untreated groups.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male Wistar rats (Simonsen, Gilroy, CA) were housed individually under controlled light (12:12 light:dark) and temperature conditions. Animals had access to food and water ad libitum. Rats were divided randomly into TZD-treated or nontreated groups and subdivided into control (saline) or Liposyn (Abbott Laboratories, Chicago) infusion groups. Initially, three animal groups were studied: 1) control untreated (n = 7), 2) intralipid infused (FFA1, n = 7), and 3) troglitazone-treated + intralipid infused (TZD + FFA1, n = 7).
TABLE 1
Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FFA1</th>
<th>TZD + FFA1</th>
<th>TZD + FFA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>0.300 ± 0.015</td>
<td>0.328 ± 0.012</td>
<td>0.292 ± 0.016</td>
<td>0.324 ± 0.010</td>
</tr>
<tr>
<td>Arterial glucose (nmol/l)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>7.9 ± 0.28</td>
<td>8.3 ± 0.43</td>
<td>7.7 ± 0.48</td>
<td>6.9 ± 0.46</td>
</tr>
<tr>
<td>Clamp</td>
<td>8.38 ± 0.08</td>
<td>8.49 ± 0.12</td>
<td>8.21 ± 0.17</td>
<td>8.17 ± 0.17</td>
</tr>
<tr>
<td>Arterial insulin (nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clamp</td>
<td>6.5 ± 2.4</td>
<td>7.2 ± 3.4</td>
<td>7.2 ± 1.3</td>
<td>8.2 ± 2.7</td>
</tr>
<tr>
<td>Basal HGO (ng · kg⁻¹ · min⁻¹)</td>
<td>11.9 ± 1.2</td>
<td>12.4 ± 1.5</td>
<td>9.3 ± 0.97</td>
<td>9.1 ± 0.72</td>
</tr>
</tbody>
</table>

No significant differences observed between groups.

RESULTS

Twenty days of TZD treatment had no impact on basal metabolic parameters (glucose, FFA, or HGO) or body weight (mean = 0.311 ± 0.013 kg; P = 0.08; Table 1). During the clamp, insulin concentrations reached a plateau of 7.3 ± 2.45 mmol/l for all groups, whereas arterial glucose was tightly clamped at 8.31 ± 0.14 mmol/l, with no significant differences observed between the groups.

Liposyn infusion caused FFA concentrations to rise very rapidly and significantly (FFA1; basal = 0.65 ± 0.05 mmol/l vs. clamp = 2.96 ± 0.25 mmol/l; P < 0.05) in non-TZD-treated rats (Fig. 1). FFA concentrations in TZD-pretreated animals also rose significantly above basal during Liposyn infusion; however, the steady-state concentration remained significantly lower (1.8 ± 0.27 mmol/l; P < 0.007) than in untreated animals (Fig. 1). Because of this difference in arterial FFA concentration between treated and untreated animals, an additional group of TZD-pretreated animals (TZD + FFA2) was added to the investigation. This fourth group was infused with Liposyn at a rate 44% higher than FFA1, yielding a steady-state clamp FFA concentration matched identically (2.9 ± 0.28 mmol/l) to that of untreated animals (FFA1). As expected, insulin infusion alone (control clamp) caused a significant reduc-
tion in arterial plasma FFA (basal = 0.68 ± 0.06 mmol/l vs. clamp = 0.17 ± 0.02 mmol/l).

The insulin-stimulated GDR increased significantly above basal, by 35.9 ± 1.5 mg · kg⁻¹ · min⁻¹, for control animals during the clamp. Liposyn infusion caused a significant blunting (40%) in the exogenous glucose requirement (Fig. 2A) and in the maximum insulin-stimulated GDR (FFA1 = 22.7 ± 3.5; P = 0.001; Fig. 2B). Troglitazone pretreatment prevented acute Liposyn-induced decrement in insulin-stimulated GDR. The values for both TZD-treated groups (TZD + FFA1 = 41 ± 3.6 mg · kg⁻¹ · min⁻¹; TZD + FFA2 = 45.4 ± 1.7 mg · kg⁻¹ · min⁻¹) were identical to that of controls (Fig. 2B).

HGO was significantly suppressed (84 ± 4%) from 11.9 ± 1.2 (basal) to 1.8 ± 0.7 mg · kg⁻¹ · min⁻¹ during the hyperinsulinemic clamp in control animals (Fig. 2C). The suppressive effect of insulin on the liver was significantly blunted in the untreated Liposyn-infused rats (5.1 ± 1.0 mg · kg⁻¹ · min⁻¹; 58 ± 8%; P = 0.03 vs. control). TZD pretreatment reversed the FFA (FFA1) effect on HGO during maximum insulin stimulation (TZD + FFA1 vs. FFA1; P = 0.043). There was no difference in percentage suppression in HGO between control and TZD + FFA1 (72 ± 6%; P = 0.07). In TZD-treated animals that received Liposyn at an elevated rate (FFA2) compared with FFA1, the suppressive effect of insulin on HGO was significantly blunted (49 ± 14% suppression from basal) compared with controls. Although insulin was less effective at suppressing HGO in TZD + FFA2 compared with TZD + FFA1, the values did not reach statistical significance (P = 0.1).

In addition, we examined the effects of elevated FFA levels on some of the early steps in insulin action in skeletal muscle samples. To accomplish this, we obtained skeletal muscle samples from all animals at the end of the hyperinsulinemic-euglycemic clamp; thus, these samples represent the fully insulinized state, which existed at the termination of the glucose clamp study. Insulin receptor tyrosine phosphorylation assessed by phosphotyrosine immunoblotting was the same in all groups (data not shown). In contrast, we found a 25% decrease (P < 0.05) in IRS-1 tyrosine phosphorylation in control Liposyn-infused animals, whereas no change in IRS-1 phosphorylation existed in the TZD-pretreated groups (Fig. 3A and B). Total IRS-1 protein levels were the same in all groups (Fig. 3C and D). We also measured IRS-1–associated PI 3-kinase activity in these muscles and found a 40% decrease in Liposyn-infused animals, which was prevented in the TZD-pretreated groups (Fig. 4). Thus, elevated FFA levels were associated with a decrease in insulin signaling downstream of the receptor, and this effect was completely prevented by TZD pretreatment.

Skeletal muscle fatty acid translocase (FAT/CD36) total protein was significantly reduced (50%; P = 0.001) in untreated animals after 2 h of Liposyn infusion (Fig. 5). In contrast, total FAT protein in TZD-pretreated animals (TZD + FFA1, TZD + FFA2) was identical to control animals despite elevated FFA concentrations similar to those observed in untreated animals (FFA1; Fig. 5). It is interesting that results from Western blot analysis of FAT/CD36 total protein paralleled those for GDR. No difference in muscle FAT/CD36 total protein content, taken from a separate group of TZD-treated and untreated rats under basal conditions, was detected (data not shown). These data suggest that troglitazone treatment protects FAT/CD36 from an FFA-induced reduction. We were unable to determine whether TZD pretreatment protected FAT/CD36 from degradation, a reduction in protein synthesis, or a combination of both processes.

**DISCUSSION**

It is widely known that elevated FFA levels can exert a deleterious effect on insulin’s overall actions, and this has been demonstrated in both animals and humans (1–3). Thus, artificial elevation of circulating FFA levels impairs the ability of insulin to stimulate overall body glucose disposal and also interferes with insulin’s ability to inhibit hepatic glucose production (6,19,20). Although there is significant

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**FIG. 1.** FFA concentrations measured at basal and during hyperinsulinemia. Values are expressed as means ± SE for the four experimental groups: control, intralipid infused (FFA1), TZD-treated + intralipid infused (TZD + FFA1), and TZD-treated + intralipid infused 2 (TZD + FFA2; increased intralipid infusion rate). *Significance versus basal (P < 0.05); #significance versus control (P < 0.05); †significance versus FFA1 (P < 0.05).
In the current study, we confirmed that elevations of FFA levels in normal male rats lead to peripheral as well as hepatic insulin resistance. In addition, we showed that pretreatment of these animals with the insulin sensitizing TZD troglitazone completely prevents the adverse effects of excess FFAs on insulin action. We also showed that elevated FFA levels lead to a striking decrease in skeletal muscle IRS-1 phosphorylation and PI 3-kinase activation and that these defects in insulin signaling are entirely prevented by treatment with the TZD. Thus, the preventive effects of TZDs on lipotoxicity-induced insulin resistance are well correlated with improved skeletal muscle insulin signaling through the IRS-1/PI 3-kinase mechanism.

Although the mechanisms of TZD-induced insulin sensitization in vivo are not well known, it has been suggested that these agents improve insulin action in skeletal muscle secondarily by exerting primary effects on adipose tissue (21). One concept to explain this is that TZD treatment lowers circulating FFA levels, which then leads to improved insulin action in skeletal muscle. Such an explanation would not hold for the current results, as our data argue that a TZD-induced decrease in FFA levels may have little to do with the overall effects of these agents to improve insulin sensitivity. Thus, in these studies, the circulating FFA levels were fixed through the infusion of Liposyn plus heparin and were the same between the control non–TZD-treated animals and the TZD-treated animals that were infused with Liposyn at a higher rate (TZD + FFA2). Because circulating FFA levels were the same between these two groups, whereas the TZD-treated animals did not develop insulin resistance as did controls, it follows that the mechanisms of TZD-induced insulin sensitization in this setting cannot be explained by decreased FFA levels. In fact, to match circulating FFA levels, we had to infuse the TZD-treated animals with 44% more Liposyn, indicating that the overall FFA flux and metabolism were greater in the TZD-treated group.

Although TZD-induced lowering of FFA levels is not the cause of insulin sensitization in our model, our results do provide some insight into potential mechanisms of the beneficial effects of TZDs on insulin action. During the lipid infusions in non–TZD-treated animals, we noted a marked decrease in insulin-stimulated tyrosine phosphorylation in skeletal muscle IRS-1, accompanied by a striking decrease in insulin-stimulated PI 3-kinase activity. An FFA-induced decrease in PI 3-kinase activity was reported previously by Griffin et al. (22), and our data support the physiologic relevance of this finding. Importantly, in the TZD-treated animals, there was no FFA-induced decrease in IRS-1 phosphorylation or PI 3-kinase activity. That elevated FFAs cause insulin resistance as well as a decrease in IRS-1/PI 3-kinase and insulin sensitivity provides strong correlative evidence that the changes in IRS-1/PI 3-kinase and insulin sensitivity are related phenomena. However, correlative results do not necessarily prove causality, and it remains possible that the changes in insulin sensitivity and IRS-1/PI 3-kinase activity represent independent effects of these agents, which are not mechanistically connected (23). Jucker et al. (24) demonstrated that elevated FFAs cause insulin-stimulated glucose transport, and our current data provide a plausible explanation for such an effect. This concept...
would be different from the original effects proposed by Randle et al. (25) and provide a more focused explanation for insulin resistance associated with lipotoxicity.

Another possible aspect of lipotoxicity concerns intramyocyte triglyceride deposition. It has been noted that insulin resistance in rodents and humans is associated with increased triglyceride deposition within skeletal muscle cells (26,27). However, it is not known whether this increase in triglyceride deposition is related to elevated circulating FFA levels or triglyceride deposition reflects some other defect. Although a mechanism linking triglyceride deposition to insulin resistance has yet to be defined, it is interesting to note that TZD treatment can lead to a decrease in muscle triglyceride content (28). Whether this is related to the TZD effect of preventing FFA-induced insulin resistance is unknown.

The immediate target for TZD action is the PPAR-\( \gamma \) receptor (29,30). This receptor is expressed predominantly in adipose tissue, but several studies also have demonstrated PPAR-\( \gamma \) expression in skeletal muscle, at ~10% the level of adipose tissue (31,32). Because skeletal muscle accounts for the great majority of insulin-stimulated glucose disposal, it follows that the defects that cause impaired in vivo glucose disposal must be manifested in skeletal muscle. This then leads to the question of whether TZDs exert their insulin-sensitizing effects directly on skeletal muscle or work indirectly by secondary mechanisms. For example, TZDs work directly on adipose tissue, and it is possible that adipocytes convey some signal to muscle that leads to insulin sensitization. It would seem that the results from the current experiments are more consistent with a direct action of TZDs on skeletal muscle.

FIG. 3. A: Skeletal muscle (red quadriceps) IRS-1 tyrosine phosphorylation immunoblot reflecting three animals per group. B: Mean ± SE values for IRS-1 phosphorylation for the four experimental groups are represented in graph form below the immunoblot. Means reflect six animals per group and are displayed in arbitrary units. *Significance versus control. C: Antiphosphotyrosine blots were stripped and rebotted with anti-IRS-1 antibody to quantify total IRS-1 protein. The immunoblot (C) reflects three animals per group. D: Mean ± SE values for IRS-1 total protein for the four experimental groups are represented in graph form below the immunoblot. Means reflect six animals per group and are displayed in arbitrary units. *Significance versus control.

FIG. 4. IRS-1–associated PI 3-kinase activity was assessed in red quadriceps muscle for the four experimental groups. Values are expressed as mean ± SE for six animals per group and displayed in arbitrary units. *Significance versus control (\( P < 0.05 \)).
Because steady-state plasma FFA levels were lower in TZD-treated rats during intralipid infusion (FFA1), one can conclude that FFA clearance was increased in these animals compared with untreated control animals. This is supported by the fact that the intralipid infusion rate required to produce identical plasma FFA concentrations between TZD-treated and untreated animals was elevated by 44% in TZD-treated animals. These findings are consistent with the fact that FAT/CD36 was strikingly reduced in untreated versus TZD-treated groups. Because FAT/CD36 is a primary transporter of fatty acids into skeletal muscle, a reduction in the amount of FAT/CD36 protein would produce a decrease in fatty acid uptake. Furler et al. (40) speculated, on the basis of their findings, that nonesterified fatty acid (NEFA) influx into white adipose beds represents only 3–5% of whole-body NEFA turnover after a 5- and 36-h fast, respectively. These ideas also are consistent with the recent work of Febbraio et al. (41) in FAT/CD36 null mice. Taken together, these data indicate a significant role for skeletal muscle in fatty acid metabolism. Because there is a significant relationship between intramyocellular triglyceride concentration and insulin resistance, the ability of skeletal muscle to process NEFA seems to be of great importance.

In summary, the current studies show that elevated circulating FFA levels lead to skeletal muscle and hepatic insulin resistance and that this is associated with corresponding decreases in insulin-signalizing events. Treatment of animals with a TZD prevents FFA-induced insulin resistance in skeletal muscle and maintains normal activity of the insulin-signalizing events that we measured. These TZD effects are unrelated to changes in FFA levels and may involve direct effects of these agents on skeletal muscle insulin signaling and FFA partitioning.

ACKNOWLEDGMENTS
This work was supported by a research grant from the National Institutes of Health (DK-33651) and the Veterans Administration San Diego Health Care System, Research Service. A.L.H. is supported by research training grants from the National Institutes of Health (DK-07494 and DK-33649).

We express our appreciation and gratitude to Dr. Ted Ciaraldi and Dr. Lorraine Turcotte for technical expertise and helpful discussions related to FFA metabolism.

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