Elevated Toll-Like Receptor 4 Expression and Signaling in Muscle From Insulin-Resistant Subjects

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OBJECTIVE—Toll-like receptor (TLR)4 has been implicated in the pathogenesis of free fatty acid (FFA)-induced insulin resistance by activating inflammatory pathways, including inhibitor of κB (IkB)/nuclear factor κB (NFκB). However, it is not known whether insulin-resistant subjects have abnormal TLR4 signaling. We examined whether insulin-resistant subjects have abnormal TLR4 expression and TLR4-driven (IkB/NFκB) signaling in skeletal muscle.

RESEARCH DESIGN AND METHODS—TLR4 gene expression and protein content were measured in muscle biopsies in 7 lean, 8 obese, and 14 type 2 diabetic subjects. A primary human myotube culture system was used to examine whether FFAs stimulate IkB/NFκB via TLR4 and whether FFAs increase TLR4 expression/content in muscle.

RESULTS—Obese and type 2 diabetic subjects had significantly elevated TLR4 gene expression and protein content in muscle. TLR4 muscle protein content correlated with the severity of insulin resistance. Obese and type 2 diabetic subjects also had lower IkBα content, an indication of elevated IkB/NFκB signaling. The increase in TLR4 and NFκB signaling was accompanied by elevated expression of the NFκB-regulated genes interleukin (IL)-6 and superoxide dismutase (SOD)2. In primary human myotubes, acute palmitate treatment stimulated IkBα/NFκB, and blockade of TLR4 prevented the ability of palmitate to stimulate the IkBα/NFκB pathway. Increased TLR4 content and gene expression observed in muscle from insulin-resistant subjects were reproduced by treating myotubes from lean, normal-glucose-tolerant subjects with palmitate. Palmitate also increased IL-6 and SOD2 gene expression, and this effect was prevented by inhibiting NFκB.

CONCLUSIONS—Abnormal TLR4 expression and signaling, possibly caused by elevated plasma FFA levels, may contribute to the pathogenesis of insulin resistance in humans. Diabetes 57:2595–2602, 2008
The over-aging of a lipid infusion to activate the IkB/NFkB pathway in adipose tissue (14) and to inhibit insulin-stimulated whole-body glucose disposal (14,17) is reduced in TLR4 null mice. Because muscle is the major site of insulin-stimulated glucose disposal (18), this important finding implies that TLR4 is also functional in skeletal muscle. Indeed, muscles from mice carrying an inactivating mutation on TLR4 are prevented from developing lipid-induced insulin resistance (16). Whereas these data obtained from animal and tissue culture models clearly implicate TLR4 in the pathogenesis of insulin resistance, it remains unknown whether this pathway functions abnormally and contributes to insulin resistance in muscle from human subjects. Therefore, the purpose of this study was to examine whether insulin-resistant subjects have abnormal TLR4 expression and signaling in muscle and to determine whether TLR4 plays a role in the stimulation of IkB/NFkB caused by FFAs. We hypothesize that 1) insulin-resistant subjects have abnormal function of TLR4 in muscle, and 2) TLR4 is involved in FFA-induced IkB/NFkB stimulation in human muscle.

**RESEARCH DESIGN AND METHODS**

We recruited 7 lean, 8 obese nondiabetic, and 14 obese subjects with type 2 diabetes. All subjects were sedentary and had stable body weight for 6 months before study. Each subject underwent a medical history, physical examination, screening laboratory tests, and an oral glucose tolerance test (OGTT). Lean and obese nondiabetic subjects had no family history of type 2 diabetes and were normal glucose tolerant. None of the lean or obese subjects were taking any medication. Three type 2 diabetic subjects were taking glipizide, which was withdrawn 3 days before the OGTT. Nine type 2 diabetic subjects were treated with diet alone. Other than glipizide, no subject was taking any medication known to affect glucose metabolism. The study was approved by the institutional review board of the University of Texas Health Science Center at San Antonio (UTHSCSA), and all subjects gave written consent. OGTT. Plasma glucose and FFA levels were measured at baseline and every 15 min for 2 h after the ingestion of 75 g glucose. Plasma insulin was measured at baseline, and the severity of insulin resistance was estimated using the homeostasis model assessment (HOMA) index (19).

**Muscle biopsies.** Subjects reported to the clinical research center at 8:00 a.m. after an overnight fast and refrained from any exercise for 48 h before the muscle biopsy. Subjects rested for 30 min before a vastus lateralis muscle biopsy (20). The muscle was debrided of adipose and connective tissue and immediately (within 5 s after the biopsy) frozen in liquid nitrogen.

**Generation of primary myotubes.** Primary skeletal muscle cells were grown from satellite cells obtained from the muscle tissue based on the G9251B protocol, with mitogens added and a normal glucose tolerance test. After excision, the muscle tissue (~100 mg) was immediately placed in 15 ml Ham’s F-12 medium at 4°C. After removing excess fat and connective tissue, the samples were digested in 0.05% trypsin, 0.1% collagenase, and 1.5% BSA, pH 7.4, for 30 min at 37°C with agitation. After 30 min of incubation, 10% FBS was added and the tissue was centrifuged at 80 g for 10 min. Cell pellets were resuspended with 200 μg/ml α-minimal essential medium (MEM) containing penicillin (200 units/ml)/streptomycin (200 μg/ml)/streptomycin (200 μg/ml). Cells were plated on 100-mm dishes and incubated for 1 h to remove fibroblasts. The myoblasts then were grown to confluence in 75 cm² collagen-coated flasks with 20% α-MEM for 5–7 days and subsequently seeded in 75 cm² flasks for another 5 days. For all individual experiments, myoblasts were seeded in six-well culture dishes (9.6 cm²/well) at a density of 20,000 cells/well. When myoblasts reached 80–90% confluence, the cells were fused for 4–7 days in α-MEM with 2% FBS. Cells displayed the typical features of differentiated myotubes: elongated, multinucleated cells expressing myosin heavy chain by Western blotting.

**Microscopy.** To examine the effect of palmitate on IL-6, SOD2, and TLR4 gene expression and protein levels in human muscle, myotubes then were lysed, and TLR4 protein content was measured by Western blotting.

**Elevated TLR4 expression and insulin resistance**

To examine whether insulin-resistant subjects have abnormal TLR4 expression and signaling in muscle and to determine whether TLR4 plays a role in the stimulation of IkB/NFkB caused by FFAs. We hypothesize that 1) insulin-resistant subjects have abnormal function of TLR4 in muscle, and 2) TLR4 is involved in FFA-induced IkB/NFkB stimulation in human muscle.
Elevated TLR4 gene expression and protein content in obese and type 2 diabetic subjects. TLR4 gene expression was significantly increased in the obese nondiabetic and type 2 diabetic subjects (4.8- and 3.2-fold, respectively; \( P < 0.05 \)) compared with lean subjects (Fig. 1A). Collectively, in the three groups, absolute TLR4 mRNA levels correlated with fasting plasma FFA concentrations (\( r = 0.37, P = 0.05 \)) and FFA AUC during OGTT (\( r = 0.41, P = 0.03 \)). To examine whether increased TLR4 gene expression in obese and type 2 diabetic subjects was reflected at the protein level, we measured TLR4 muscle protein content by Western analysis using an antibody targeted against the epitope corresponding to amino acids 242–321 of the internal region of human TLR4. Using this antibody, TLR4 migrated as a single ~100-kDa band. To confirm the specificity of the antibody, human embryonic kidney cell lysates overexpressing human TLR4 tagged with yellow fluorescent protein (provided by Dr. Andrei Medvedev, University of Maryland) were immunoprecipitated with an anti-GFP antibody (Invitrogen, Carlsbad, CA) and blotted with anti-TLR4 (not shown). Consistent with the increases in TLR4 gene expression, obese and type 2 diabetic subjects had 2.8- and 2.2-fold higher TLR4 muscle protein content than lean subjects (\( P < 0.05 \)) (Fig. 1B), and there was a positive correlation (\( r = 0.57, P = 0.003 \)) between the HOMA index of insulin resistance (HOMA-IR) and TLR4 content (Fig. 1C).

Similar to TLR4, the gene expression of TLR2 was significantly elevated in muscle from obese and type 2
diabetic individuals (P < 0.05) (Fig. 1D). Nonetheless, we were not able to detect TLR2 protein in muscle from human subjects (diabetic and nondiabetic) by direct Western blotting, despite using several commercially available antibodies. Figure 1E shows that, in contrast to muscle, human mononuclear cells highly express TLR2 protein.

**TLR4-driven signaling.** We examined whether increased TLR4 gene expression and protein content in insulin-resistant subjects was associated with abnormal TLR4 signaling by measuring abundance of IkBα. Obese and type 2 diabetic subjects had decreased IkBα content in muscle (Fig. 2A). Because phosphorylation of IkB by IKK leads to IkB degradation, a reduction in IkB abundance is considered to indicate activation of the IkB/NFκB pathway (31). IkB abundance inversely correlated with fasting plasma FFA concentrations (r = −0.6, P = 0.005) and FFA AUC during OGTT (r = −0.46, P = 0.03) and tended to correlate negatively with the HOMA index (r = −0.34, P = 0.1). Currently, we are investigating whether direct measurement of IKK activity (which was not measured because of insufficient protein) has a stronger (negative) correlation with insulin sensitivity measured with the insulin clamp.

We also determined the expression of IL-6 and SOD2, genes that are highly regulated by NFκB (12,13), in muscle from obese and type 2 diabetic subjects. As shown in Figs. 2B and C, obese and type 2 diabetic subjects had elevated IL-6 and SOD2 gene expression compared with lean normal-glucose-tolerant subjects (P < 0.05). Collectively, these results indicate that insulin-resistant subjects have increased TLR4 expression/content and TLR4-driven signaling.

**FFAs acutely stimulate TLR4-driven signaling.** To explore whether FFAs directly stimulate TLR4-driven signaling in human muscle, we used a primary muscle cell culture system using satellite cells obtained from lean, normal-glucose-tolerant subjects. Treatment with 200 and 400 µmol/l palmitate decreased IkBα abundance (Fig. 3A) and increased IkB phosphorylation within 15 min (Fig. 3D). Because phosphorylation of IkB by IKK leads to IkB degradation, a reduction in IkB abundance is considered to indicate activation of the IkB/NFκB pathway (31). IkB abundance inversely correlated with fasting plasma FFA concentrations (r = −0.6, P = 0.005) and FFA AUC during OGTT (r = −0.46, P = 0.03) and tended to correlate negatively with the HOMA index (r = −0.34, P = 0.1). Currently, we are investigating whether direct measurement of IKK activity (which was not measured because of insufficient protein) has a stronger (negative) correlation with insulin sensitivity measured with the insulin clamp.

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3B), an effect that persisted after 60 and 240 min of palmitate treatment. Treatment with 100 μmol/l palmitate for up to 240 min did not consistently increase IκB phosphorylation (not shown). In line with the increases in IκB phosphorylation, palmitate significantly increased IKK kinase activity (Fig. 3C). Stearate, another saturated fatty acid, also stimulated IκB/NFκB signaling, whereas the unsaturated fatty acid linoleate did not affect this pathway (Fig. 3D). This finding indicates that the stimulatory effect of palmitate on TLR4-driven signaling in human muscle is common to other saturated fatty acids.

**TLR4 mediates the effect of palmitate on IκB/NFκB signaling.** The role of TLR4 in the activation of IκB/NFκB by FFAs was assessed by blocking TLR4. Preincubation of human myotubes with TLR4 neutralizing antibodies prevented the ability of palmitate to phosphorylate IκB (Fig. 4A), whereas antibodies against TLR2 did not block the stimulatory effect of palmitate (Fig. 4B).

**Prolonged FFA exposure increases TLR4 expression.** Because plasma FFA, insulin, TNF-α, and IL-6 concentrations are typically elevated in insulin-resistant subjects, we examined whether these factors had any effect on muscle TLR4 content. Figure 5A shows that a 3-day exposure to palmitate caused a significant increase in TLR4 protein content in human myotubes. In contrast to palmitate, chronic exposure to insulin, TNF-α, or IL-6 did not increase TLR4 protein content (not shown). Consistent with the elevation in TLR4 protein content, treatment with palmitate for 6 h also increased TLR4 gene expression (Fig. 5B). Importantly, this effect was reproduced by stimulating the cells with synthetic monophosphoryl lipid A (Fig. 5C), a specific TLR4 agonist (32).

**The proinflammatory effect of palmitate is NFκB mediated.** An IκB superrepressor mutant was used to examine whether the effect of palmitate on IL-6, SOD2, and TLR4 mRNA expression is mediated by NFκB. As shown in...
Fig. 6, palmitate decreased IκBα abundance (Fig. 6A), stimulated NFκB activity (Fig. 6B), and increased the gene expression of IL-6 (Fig. 6C) and SOD2 (Fig. 6D), effects that were blocked by the IκB supperrepressor. Palmitate-induced TLR4 gene expression was also blocked by inhibiting NFκB through overexpression of the IκB supperrepressor (Fig. 6E), but not by preincubating the cells with TLR4-neutralizing antibodies (not shown).

DISCUSSION

The main finding of this study is that obese and type 2 diabetic subjects have increased TLR4 gene expression/content and TLR4-driven (IκB/NFκB) signaling in skeletal muscle. To examine whether FFAs directly activate TLR4 signaling in human muscle, myotubes were treated with palmitate, which rapidly stimulated the IκB/NFκB cascade. Furthermore, blocking TLR4 completely inhibited the ability of palmitate to stimulate this pathway. Because most obese nondiabetic and type 2 diabetic subjects have elevated plasma FFA concentrations (33), our results suggest that TLR4 may play an important pathogenic role in the mechanism by which FFAs induce an inflammatory response in insulin-resistant subjects.

To elucidate the cause for the elevation in TLR4 content present in the muscle from the obese and type 2 diabetic subjects, human myotubes were treated with palmitate for 3 days. Chronic palmitate treatment caused a significant increase in myotube TLR4 content, whereas prolonged exposure to insulin, TNF-α, or IL-6, whose plasma concentrations are typically elevated in insulin-resistant (obese and type 2 diabetic) subjects, had no effect. In line with this finding, palmitate increased TLR4 gene expression, an effect that was mediated via NFκB. Consistent with investigations performed in human endothelial cells that showed that LPS increases TLR4 expression (34), we found that treatment with the specific TLR4 agonist monophosphoryl lipid A also induces TLR4 gene expression in human myotubes. This suggests that palmitate-induced TLR4 gene expression is dependent on TLR4 activation. Nonetheless, this effect was not inhibited by TLR4-neutralizing antibodies, suggesting that, in addition to TLR4 stimulation, other mechanisms, such as accumulation of intracellular lipids (ceramides and diacylglycerol), may be involved in FFA-induced TLR4 expression. Because the elevations in TLR4 expression/content in the muscle from insulin-resistant subjects were reproduced in myotubes by prolonged exposure to palmitate, one could speculate that these increases in
TLR4 expression/content are acquired defects, secondary to excess FFA supply. Nonetheless, future studies will be needed to establish whether there also is a genetic (inherited) basis underlying the elevation in TLR4 expression in insulin-resistant muscle.

Senn (35) reported that TLR2, another member of the TLR family, is involved in palmitate-induced insulin resistance in mouse-derived C2C12 myotubes. To examine this possibility in humans, we measured TLR2 gene expression in muscle from insulin-resistant subjects. Both obese and type 2 diabetic individuals displayed elevated TLR2 gene expression in muscle. However, TLR2 protein was not detected in muscle by direct Western blotting, although a signal (too weak for quantitation) corresponding to TLR2 was detected after immunoprecipitation. Taking into account that absolute mRNA levels of TLR4 versus TLR2 in human muscle were roughly similar (not shown), the low TLR2 protein content observed in human muscle is likely due to post-transcriptional regulation and/or protein instability (i.e., degradation). Poor antibody immunoreactivity due to post-transcriptional regulation and/or protein instability to TLR2 protein observed in human muscle, although this seems unlikely considering that TLR2 protein was readily detectable in human mononuclear cells. Importantly, TLR2 neutralizing antibodies did not affect the ability of palmitate to activate IkB/NFkB signaling. Based on these results, it is unlikely that TLR2 plays a major role in lipid-induced insulin resistance in human muscle.

Another relevant finding in this study is the increased gene expression of the inflammatory proteins IL-6 and SOD2 in muscle from obese and type 2 diabetic subjects. Experiments performed in monocyte-like (U937 cells (12) and fibroblasts (13) have demonstrated that the expression of these proteins is regulated by NFkB. Human myotubes were treated with palmitate to explore whether elevated circulating FFA levels in obese and type 2 diabetic subjects might be responsible for the increased gene expression of IL-6 and SOD2 in muscle. Palmitate robustly increased mRNA expression of IL-6 and SOD2 in the myotubes. Moreover, blockade of NFkB completely blocked the ability of palmitate to induce the gene expression of these inflammatory proteins. Therefore, elevation in plasma FFA concentration may contribute to increased gene expression of IL-6 and SOD2 in muscle from insulin-resistant subjects, and this effect is likely mediated by NFkB.

Accumulating evidence suggests that monocytes/macrophages play an important role in the pathogenesis of insulin resistance by infiltrating insulin-sensitive tissues, such as muscle and fat (36,37). Thus, it is possible that the increases in TLR4 expression and signaling observed in the muscle tissue from insulin-resistant subjects are due to inflammatory cell infiltration of the muscle, rather than from intrinsic upregulation of TLR4 expression/signaling within the myofibers. For this reason, we performed Western analysis and real-time PCR of EMR1, a marker for monocyte/macrophage infiltration, in muscle tissue of insulin-resistant subjects and in cultured myotubes. Unlike CD14+ monocytes (AllCells, Emeryville, CA), which expressed EMR1, neither EMR1 protein nor mRNA was detected in the muscle tissue or myotubes (not shown). It is therefore unlikely that the differences in TLR4 expression/signaling observed between groups are secondary to monocyte/macrophage infiltration of muscle.

In conclusion, insulin-resistant subjects have increased TLR4-driven signaling in muscle. We propose a model in which elevated plasma FFA levels in obese and type 2 diabetic subjects activates this signaling pathway by 1) directly interacting (binding) with TLR4 and 2) increasing TLR4 gene expression and protein content, leading to a net increase in the number of TLR4 receptors available for stimulation by elevated plasma FFA levels. Strategies aimed at reducing TLR4 expression, or at blocking TLR4 signaling, may prove useful in enhancing insulin sensitivity in insulin-resistant individuals.

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