Toll-like receptor 2 mediates high-fat diet-induced impairment of vasodilator actions of insulin

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1Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, 2Department of Cell, Developmental, and Integrative Biology, and 3University of Alabama at Birmingham Comprehensive Diabetes Center, University of Alabama at Birmingham, Birmingham, Alabama; 4Western Human Nutrition Research Center, United States Department of Agriculture and Department of Nutrition, University of California-Davis, Davis, California; and 5Department of Medicine, Division of Endocrinology, Diabetes, and Nutrition, University of Maryland School of Medicine, Baltimore, Maryland

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Jang H, Kim H, Hwang DH, Quon MJ, Kim J. Toll-like receptor 2 mediates high-fat diet-induced impairment of vasodilator actions of insulin. Am J Physiol Endocrinol Metab 304: E1077–E1088, 2013. First published March 26, 2013; doi:10.1152/ajpendo.00578.2012.—Obesity is characterized by a chronic proinflammatory state that leads to endothelial dysfunction. Saturated fatty acids (SFA) stimulate Toll-like receptors (TLR) that promote metabolic insulin resistance. However, it is not known whether TLR2 mediates impairment of vascular actions of insulin in response to high-fat diet (HFD) to cause endothelial dysfunction. siRNA knockdown of TLR2 in primary endothelial cells opposed palmitate-stimulated expression of proinflammatory cytokines and splicing of X box protein 1 (XBP-1). Inhibition of unfolding protein response (UPR) reduced SFA-stimulated expression of TNFα. Thus, TLR2 mediates UPR and proinflammatory response through activation of TLR2 in endothelial cells. Knockdown of TLR2 also opposed impairment of insulin-stimulated phosphorylation of eNOS and subsequent production of NO. Importantly, insulin-stimulated vasorelaxation of mesenteric arteries from TLR2 knockout mice was preserved even on HFD (in contrast with results from arteries examined in wild-type mice on HFD). We conclude that TLR2 in vascular endothelium mediates HFD-stimulated proinflammatory responses and UPR that accompany impairment of vasodilator actions of insulin, leading to endothelial dysfunction. These results are relevant to understanding the pathophysiology of the cardiovascular complications of diabetes and obesity.

endothelial function; vascular insulin resistance; Toll-like receptor 2; unfolding protein response; inflammation; endothelial nitric oxide synthase

INSULIN SIGNALING PATHWAYS involving insulin receptor/insulin receptor substrates (IRS)/phosphatidylinositol (PI) 3-kinase/protein kinase B (Akt) contribute to glucose uptake in skeletal muscle and adipose tissue. A similar insulin-signaling pathway in vascular endothelium leads to activation of endothelial nitric oxide synthase (eNOS) and production of nitric oxide (NO), which contribute to vasodilation and capillary recruitment (18). Vasodilator actions of insulin play important roles in antiatherogenesis and capillary recruitment that regulate hemodynamics as well as nutrient metabolism (23, 51). Impairment of vascular actions of insulin leads to a reduction of eNOS activity and endothelial dysfunction that contributes to both metabolic dysfunction and cardiovascular complications. This is evident from the metabolic and cardiovascular phenotype of eNOS knockout mice (9, 18, 43). High-fat diet (HFD) promotes proinflammatory responses and endoplasmic reticulum (ER) stress, major mechanisms for obesity-induced impairment of insulin actions (13, 18, 46). Unfolding protein response (UPR) is a cellular protective mechanism that copes with ER stress by reducing protein synthesis, inducing expression of chaperones, and stimulating protein degradation. UPR is stimulated by a bacterial infection that increases proinflammatory responses as a host-protective mechanism (30). Toll-like receptors (TLR) are pattern-recognition receptors that detect pathogen-associated molecular patterns and induce innate immune responses for host defense (22, 50). TLR4 recognizes lipopolysaccharide from gram-negative bacteria, whereas TLR2 recognizes bacterial lipoproteins. Triacylated lipopeptide activates the TLR2/1 heterodimer, and diacylated lipopeptide activates the TLR2/6 heterodimer (3, 44). TLRs can be activated by various endogenous molecules, including saturated fatty acids (SFA). SFA-activated TLRs stimulate proinflammatory responses, including production of cytokines and activation of c-Jun NH2-terminal kinase (JNK) and IκB, leading to metabolic insulin resistance (21, 26, 33, 47). Indeed, TLR2- or TLR4-deficient mice are protected from HFD-induced insulin resistance (33, 48). Moreover, TLR4 mediates SFA-stimulated impairment of insulin signaling and NO production in vascular endothelial cells (16). However, the role of SFA-activated TLR2 in vasodilator actions of insulin with regard to UPR-mediated inflammatory responses is not known. In this study, we examine the role of TLR2 to promote SFA-induced impairment of insulin-stimulated vasodilation through a UPR-mediated mechanism. Moreover, we examine vasodilator actions of insulin ex vivo in intact vessels isolated from wild-type (WT) and TLR2-knockout mice.

MATERIALS AND METHODS

Materials. Anti phospho-(p)-eNOS, anti-p-Akt, anti-p-NF-κB, and anti-Akt were obtained from Cell Signaling Technology (Beverly, MA). Anti-eNOS and anti-p-JNK were obtained from Invitrogen (Carlsbad, CA), and anti-p65/NF-κB anti-JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). siRNA for human TLR2 and scrambled siRNA were purchased from Dharmacon. dsiRNA for bovine TLR2 and nontargeted scrambled siRNA and primers for the RT-PCR were purchased from Integrated DNA technologies (Coralville, IA).

Animals. All animal procedures were performed in accordance with the rules of and approved by the Animal Use and Care Committee at The University of Alabama at Birmingham. Both C57BL/6J (wild-type) and B6.129-Tlr2tmkir/J (TLR2KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a temperature-controlled facility with a 12:12-h light-dark cycle. At 6

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wk of age, mice were fed either chow (7917 Harlan Diet, 11% calories from fat) or HFD (5SPQ, 54% calories from fat; Test Diet Richmond, IN) for 10 wk. Body weight was measured every week. Mice were fasted overnight and anesthetized with pentobarbital sodium (50 mg/kg) before euthanization.

Quantitative magnetic resonance. These experiments were conducted by the University of Alabama at Birmingham (UAB) small animal phenotyping core facility funded by our Nutrition Obesity Research Center. In vivo body composition (total body fat and lean tissue) of mice was determined using an EchoMRI 3-in-1 quantitative magnetic resonance machine (Echo Medical Systems, Houston, TX). Quantification of results was standardized by conducting a system test using a known fat standard prior to experimental measurements being taken. Mice were weighed and then placed into a clear holding tube capped with a stopper that restricted vertical movement but allowed constant airflow. This tube was inserted into the machine, and the mouse was scanned using the normal precision mode.

Isolation of mouse primary heart endothelial cells. Mouse heart endothelial cells (MHEC) were isolated using a modification of previously described methods (28). Briefly, after 6-wk-old mice (WT or TLR2KO mice) were euthanized by isoflurane inhalation, the heart was dissected, minced, and incubated in PBS containing 2 mg/ml collagenase II (Worthington) for 45 min at 37°C. The digested tissues or TLR2KO mice were then incubated with microbeads (Dynal beads M-450; Invitrogen) coated with anti-CD31 (BD Biosciences) in PBS with 0.1% BSA at room temperature for 10 min. The anti-CD31 Dynabead-labeled endothelial cells were captured by a magnetic separator and seeded onto gelatin-coated 100-mm culture dishes in DMEM/high-glucose medium supplemented with 20% FBS, 45 μg/ml endothelial cell growth supplement, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml heparin. In this study, MHECs were used between passages 3 and 6. Cell culture and transfection. Bovine aortic endothelial cells (BAEC) were maintained in F-12K medium containing 5% fetal bovine serum (FBS), endothelial cell growth supplement (30 mg/L BD Biosciences), heparin sulfate (50 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Human aortic endothelial cells (HAEC; Lonza, Walkersville, MD) in primary culture were grown in F-12K medium containing EGM-2 Single Quot supplements (Lonza). All experiments were conducted on HAEC and BAEC before their sixth passage. BAEC were serum-starved (0.1% horse serum) overnight and then treated with BSA or BSA-conjugated palmitate at the indicated concentrations and for the times noted in the figure legends. HAEC were transiently transfected with 100 nM of siRNA duplex oligonucleotides [siRNA for TLR2, smartpool from Dharmacon, Lafayette, CO; dsRNA for X-box protein-1 (XBP-1) and bovine TLR2 from Integrated DNA Technologies, Coralville, IA], using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. siRNA sequences are described in Table 1. Two days after transfection, cells were serum-starved for 2 h and then treated with BSA or palmitate, as indicated in the figure legends.

Measurement of fasting glucose and insulin in serum. Mice were fasted overnight, and the blood was collected before euthanization. Glucose was measured with a hand-held glucometer (Alphatrak; Abbott, Abbott Park, IL) right before euthanization. The serum was collected after centrifugation (2,200 g for 10 min at 4°C) of clotted blood. Radioimmunoassay was performed to determine the serum insulin levels by using the Millipore (Billerica, MA) RIA kit. These analyses were conducted by the UAB core facility. Quantitative insulin sensitivity check index (QUICKI) was calculated as [1/log (fasting insulin (μU/ml) + log (fasting glucose (mg/dl)))] (6, 27, 41).

Preparation of palmitate. Preparation of palmitate was carried out as described by Mott et al. (31a). Briefly, 10.5% bovine serum albumin (Sigma A7511) was dissolved in 25 mM HEPES-DMEM and syringe-filtered (0.22 μM; Millipore). Sodium palmitate (100 mM) was heated until it was dissolved in water and added rapidly to warmed BSA solution. Then, this BSA-conjugated palmitate was added to reach the proper concentration of palmitate. We used endotoxin-free reagents and tested all of the reagents we used, including BSA, palmitate, media, and reagent diluents. We checked the endotoxin level of all of the reagents we used in this study with the Chromogenic Endotoxin Quantitation assay kit (Pierce). The levels were undetectable or <25 pg/ml.

Functional assessment for isolated mesenteric arterioles. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Mesenteric arterioles were excised from the animal and placed in a cooled (4°C) chamber containing dissection buffer (145 mM NaCl, 4.7 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, 1 mM NaH2PO4, 5 mM glucose, 3 mol/l MOPS buffer, 2 mM pyruvate, 0.02 mM EDTA, and 1% BSA, pH 7.4). The isolated arterioles were then cannulated with glass micropipettes with a 10-0 monofilament suture and mounted in a custom-designed tissue chamber (Living System Instrumentation, Burlington, VT). The arterioles were pressurized to 45 mmHg intraluminally with the same buffer without flow and superfused with buffer without albumin. The vessel preparations were positioned on the stage of an inverted microscope. The vessel segments were gradually warmed to 37°C during a 30-min equilibration period. After baseline diameter was established, arterioles were exposed to phenylephrine (1 μmol/l) until a maximal constriction was achieved (5 min). The vessels were subsequently stimulated with various vasodilators (10−11−10−5 mol/l, 3 min/concentration), including insulin, acetylcholine, and sodium nitroprusside. The dilator responses to insulin were observed and recorded. Measurement of vessel diameter (in μm) was performed with an electronic video caliper (Living Systems Instrumentation, St. Albans, Vermont) and recorded by using a software Lab Chart (AD instruments, Colorado Springs, CO). In some experiments, vessels were incubated with the NOS inhibitor Nω-nitro-l-arginine methyl ester (L-NAME; 100 μM) for 30 min prior to insulin stimulation.

The data are expressed as means ± SE. The vasodilator responses to insulin were calculated as percent relaxation from the maximum constriction induced by phenylephrine according to the following equation:

\[
\text{Relaxation }\% = \frac{(\text{ID}_{\text{rest}} - \text{ID}_{\text{PE}})}{(\text{ID}_{\text{w/o Ca}^{++}} - \text{ID}_{\text{PE}})} \times 100
\]

where \(\text{ID}_{\text{rest}}\) is the diameter obtained when the vessel was treated with insulin, \(\text{ID}_{\text{PE}}\) is the diameter obtained when the vessel was constricted with phenylephrine, and \(\text{ID}_{\text{w/o Ca}^{++}}\) is the maximal passive diameter observed when the vessel was fully dilated in buffer containing 2 mM EGTA and 100 μM adenosine without Ca++.

Preparation of cell lysates and immunoblotting. Before lysis, cells were briefly washed with ice-cold PBS. Cells were then scraped in lysis buffer containing 50 mM Tris (pH 7.2), 125 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM Na2VO4, 20 mM NaF, 1 mM Na pyrophosphate, and complete protease inhibitor cocktail (Roche Applied Science). Cell debris was pelleted by centrifugation of samples at 17,000 g for 10 min at 4°C. Supernatants were then boiled with Laemmle sample buffer for 5 min, and proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with specific antibodies, as described in the figures, using standard methods. Immunoblots were visualized and quantified by Image Analyzer (Vision Works LS) and the BioSpectrum Imaging System (UVP, Upland, CA).

Measurement of NO production. Production of NO was assessed using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (EMD Biosciences, Gibbstown, NJ), as described previously (17, 31). Briefly, endothelial cells were grown to 95% confluence in 24-well black plates (Denville, Metuchen, NJ) and serum-starved as indicated in the figure legends. Then, the cells were treated with BSA or palmitate. Cells were then loaded with 4,5-diaminofluorescein diacetate (final concentration, 1 μM) for 10 min at 37°C, rinsed 3
times with F-12K, and kept in the dark. Cells were then treated without or with insulin as indicated in the figure legends. After stimulation, cells were fixed in 2% paraformaldehyde for 5 min at 4°C. Fixed cells were visualized with a Zeiss inverted epifluorescence microscope (Axio Observer A1) using appropriate filters for a peak excitation wavelength of 480 nm and a peak emission wavelength of 510 nm. Images were captured and analyzed by using AV Rel 4.7 software with multichannel modules.

**Immunocytochemistry.** Cells were treated as described in the figure legends. After stimulation, cells were fixed in 4% paraformaldehyde-PBS for 10 min, and the cells were washed with PBS. Cells were then permeabilized with 0.1% Triton X-100-PBS for 5 min and washed with PBS. Cells were blocked with 3% BSA-PBS for 30 min and then incubated with anti-p65/NF-κB (sc-372; Santa Cruz Biotechnology) in 3% BSA/PBS at 4°C overnight. Cells were washed with PBS three times (5 min each) and then incubated with Alexa 555-conjugated goat anti-rabbit IgG (Invitrogen, Grand Island, NY) for 1 h at room temperature. Cells were washed with PBS three times (5 min each) and then stained with Hoechst 33342 (Invitrogen, Grand Island, NY). The image was visualized with a Zeiss inverted epifluorescent microscope (Axio Observer A1). Images were captured and analyzed by using AV Rel 4.7 software with multichannel modules.

**RT-PCR.** The cells were treated as described in the figure legends. Total RNA was prepared by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis by using an Omniscript RT kit (Qiagen, Valencia, CA). Then, the cDNA was subjected to semiquantitative PCR analysis by using a Hot Star Taq Master Mix kit (Qiagen). PCR product was visualized with fluorescent dye (Enviro-safe DNA/RNA Stain; Helixx Technologies), and the image was analyzed and quantified by Image Analyzer (Vision Works LS) and the BioSpectrum Imaging System (UVP). Black and white colors

Table 1.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>hGRP78</td>
<td>CATGCAGCGCTCTATGTCG</td>
</tr>
<tr>
<td>hxbp-1</td>
<td>CTTGAAAGACCTTCTCTTCT</td>
</tr>
<tr>
<td>hCHOP</td>
<td>TCTCTTCTTCTCTCTCTTCT</td>
</tr>
<tr>
<td>hIL-1β</td>
<td>CTCTCTTCTTCTCTCTTCT</td>
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<tr>
<td>hMCP-1</td>
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<td>hTNFa</td>
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<td>hIR-E-1α</td>
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<td>hTLR2</td>
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<td>β-Actin</td>
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<tr>
<td>bTLR2</td>
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<tr>
<td>DSI RNA</td>
<td>TCTCTTCTTCTCTCTTCT</td>
</tr>
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GRP78, glucose-regulated protein 78; XBP, X box protein; CHOP, C/EBP homologous protein; MCP-1, monocyte chemoattractant protein 1; IRE-1α, inositol-requiring protein-1α; TLR, Toll-like receptor; h, human; b, bovine.
were inverted by using the software. The primers for each gene are described in the Table 1.

**Statistical analysis.** Values are presented as means ± SE. Western blots were analyzed by one-way ANOVA. For the comparisons of vasodilatory responses, repeated measurements of two-way ANOVA combined with Bonferroni post hoc test were used.

**RESULTS**

**TLR2 mediates palmitate-stimulated proinflammatory responses.** To test whether TLR2 plays a role in production of proinflammatory cytokines by SFA in vascular endothelium, we knocked down TLR2 by transiently transfecting HAEC with TLR2 siRNA and treated the cells with palmitate. mRNA expression of proinflammatory marker genes was examined by semiquantitative RT-PCR analysis. Both TLR2 and TLR4 are expressed in HAEC. siRNA knockdown of TLR2 significantly reduced expression of TLR2 (vs. scrambled control siRNA; Fig. 1A). Interestingly, knockdown of TLR2 resulted in a slight increase in the expression of TLR4 that may be compensatory (Fig. 1A). Treatment with palmitate (200 μM, 6 h) increased mRNA expression of E-selectin, TNFα, IL-6, and IL-1β (Fig. 1). Knockdown of TLR2 opposed the palmitate-stimulated increase in the expression of E-selectin, TNFα, IL-6, and IL-1β. Therefore, knockdown of TLR2 impairs the ability of palmitate to induce proinflammatory cytokines and adhesion molecules in endothelial cells. However, we were not able to detect the induction of monocyte chemotactic protein-1 by palmitate treatment (data not shown) and expression of TLR downstream molecules, including myeloid differentiation primary response 88 and Toll-receptor-associated activator of interferon-β. Next, we examined palmitate-stimulated proinflammatory signaling pathways, including phosphorylation of JNK and NF-κB. Palmitate stimulated phosphorylation of JNK and NF-κB in endothelial cells as expected. These actions of palmitate were impaired by knockdown of TLR2.
Moreover, palmitate-stimulated nuclear translocation of NF-κB was inhibited by knockdown of TLR2 (Fig. 2E). These results suggest that TLR2 is required for palmitate-stimulated proinflammatory responses in vascular endothelial cells.

Role for TLR2 in palmitate-stimulated unfolding protein response that is associated with proinflammatory response. Inflammation is associated with obesity-induced ER stress (34). LPS-stimulated TLRs increase XBP-1 mRNA maturation without ER stress in macrophages (30). However, whether SFAs induce ER stress in endothelial cells through TLRs is not known. Palmitate-stimulated splicing of XBP-1 to XBP-1s was inhibited when TLR2 was knocked down. Under these same conditions, mRNA expression of CHOP and GRP78 was unaffected (Fig. 3, A–D). This suggests that treatment with palmitate stimulates UPR, similar to the response of bacteria-stimulated macrophages (30). The spliced form of XBP-1s is translated to a transcription factor that stimulates transcription of other proteins. Therefore, we next examined whether XBP-1s plays a role in palmitate-stimulated inflammatory response. siRNA knockdown of XBP-1 reduced TNFα expression (Fig. 3, E–G) but not other proinflammatory cytokines in endothelial cells (Fig. 3H). Targeted siRNA knockdown of inositol-requiring protein-1α, an endoribonuclease responsible for XBP-1 maturation, reduced the expression of TNFα but not IL-6 or E-selectin (Fig. 3, I–N). Taken together, these data suggest that TLR2 plays an important role in SFA-stimulated proinflammatory responses that are likely to involve a UPR-mediated mechanism.

TLR2 mediates palmitate-induced impairment of insulin signaling and action in vascular endothelium. Insulin signaling through IR/IRS-1/PI 3-kinase/Akt in vascular endothelial cells stimulates phosphorylation and activation of eNOS to produce NO (57, 58). We examined whether TLR2 is involved in SFA-mediated impairment of insulin signaling and action in vascular endothelium. siRNA knockdown of TLR2 opposed the effects of palmitate in insulin-stimulated production of NO (Fig. 2A–D).
With respect to insulin signaling, pretreatment of endothelial cells with palmitate impaired insulin-stimulated phosphorylation of eNOS and Akt. Importantly, siRNA knockdown of TLR2 opposed these pathological actions of palmitate to impair insulin signaling (Fig. 4, C–E).

Multiple inhibitory serine phosphorylation sites on IRS-1 have been implicated in decreased binding and activation of PI 3-kinase in response to insulin that may contribute to insulin resistance. Previously, we identified Ser24 on IRS-1 as a direct target of IL-1β receptor-activated kinase-1 and PKCδ, which are downstream components of TLR pathways (29, 32). Phosphorylation of IRS-1 at Ser24 impairs insulin-stimulated activation of PI 3-kinase and Akt (12, 19). Treatment of endothelial cells with palmitate increased phosphorylation of IRS-1 at Ser24. This was opposed by siRNA knockdown of TLR2 (Fig. 4F).

Thus, SFA may impair insulin signaling pathways in part by increasing serine phosphorylation of IRS-1 at Ser24 through TLR2-mediated signaling pathways. To exclude the possibility of artifacts from treating siRNA and incomplete knockdown, we tested insulin signaling in endothelial cells isolated from WT or TLR2KO mice. Treatment with palmitate inhibited insulin-stimulated phosphorylation of IRS-1 at Tyr612 and Akt at Ser473 in MHECs isolated from WT mice. Importantly, this effect of palmitate was abolished in endothelial cells isolated from TLR2KO mice (Fig. 4, H and I). Taken together, our data suggest that TLR2 plays an important role in palmitate-induced cross-talk between proinflammatory signaling and insulin signaling in endothelial cells. This may contribute substantially to...
Fig. 4. Insulin-stimulated production of nitric oxide (NO) in primary aortic endothelial cells is impaired by pretreatment with palmitate, whereas deficiency of TLR2 opposes this action of palmitate. 

A: HAEC were transiently transfected with scrambled control siRNA or siRNA specifically targeting TLR2. After cells were incubated for 48 h, cells were serum-starved for 2 h, and then cells were loaded with the NO-specific fluorescent dye DAF2-DA. Cells were pretreated with BSA or palmitate (200 µM, 6 h) and then treated without or with insulin (100 nM) for 20 min. Emission of green fluorescence is indicative of NO production. Experiments shown are representative of those that were independently repeated at least 3 times.

B: 3 microscopic fields/experiment were captured, and 10 randomly picked cells/field were averaged and subjected to statistical analysis by ANOVA (***P < 0.001).

C–G: bovine aortic endothelial cells (BAEC) were transiently transfected with DsiRNA for scrambled or TLR2. Then, cells were incubated for 48 h in regular medium and then serum-starved overnight and pretreated with BSA or palmitate (200 µM, 6 h for C–E; 30 min for F and G) and treated without or with insulin (100 nM) for 20 min (C–E). Cell lysates were subjected to immunoblotting with indicated antibodies. The demarcated lines are due to the noncontiguous lanes, but from a single gel (F).

H–I: mouse heart endothelial cells (MHEC) from C57BL/6J or TLR2KO mice were isolated. The endothelial cells were incubated in 1% FBS/F-12K media, and then cells were treated with BSA or palmitate (200 µM, 15 h), followed by insulin (100 nM, 20 min). Cell lysate were subjected to immunoblotting with indicated antibodies. The samples from wild-type (WT) and TLR2KO mice were run in parallel gels simultaneously. Values are means ± SE. Values significantly different (*P < 0.05; **P < 0.01; ***P < 0.005) from the values for the control are indicated. IRS-1, insulin receptor substrate-1; eNOS, endothelial nitric oxide synthase.
decreased NO production in response to insulin that is predicted to have adverse consequences on both metabolic and cardiovascular homeostasis.

Deficiency of TLR2 protects from HFD-induced impairment of insulin signaling and action in vascular endothelium. To test the role of TLR2 in obesity-induced endothelial dysfunction in intact vessels, C57BL/6J (WT) or TLR2KO mice were fed a normal chow diet (NCD; 10% calories from fat) or HFD (55% calories from fat) for 10 wk. Vasodilator actions of insulin were evaluated in mesenteric arteries isolated from these mice. As expected, both WT and TLR2KO mice gained more weight on HFD compared with NCD (Fig. 5). However, HFD TLR2KO had lower fasting glucose and insulin levels compared with HFD WT mice (Fig. 5, C and D). TLR2KO mice were partially protected from HFD-induced insulin resistance (when compared with WT mice) as assessed by QUICKI, a surrogate index for insulin sensitivity (Fig. 5E). Mesenteric arteries isolated from WT mice were dilated in response to insulin in a concentration-dependent manner (Fig. 6A). This was inhibited significantly by pretreatment with L-NAME (100 μM, competitive NOS inhibitor; Fig. 6A), indicating that insulin-stimulated vasodilator actions in these arteries are due predominantly to activity of NOS. Arteries isolated from HFD-WT mice had impaired insulin-stimulated vasodilation compared with vessels from the NCD group (P < 0.001; Fig. 6B). Of note, arteries isolated from TLR2KO mice on HFD had intact insulin-stimulated vasodilator responses comparable with those observed in arteries isolated from WT mice or TLR2KO mice on NCD (P > 0.05; Fig. 6B). Interestingly, in response to acetylcho-
homeostasis and cardiovascular function.

explain in part how HFD contributes to impaired metabolic

results suggest that HFD inhibits insulin-stimulated vasodilator

ex vivo experiments in cultured endothelial cells, these ex vivo

TLR2KO is unimpaired by HFD. Thus, consistent with our in

strongly that vascular smooth muscle function in both WT and

vessels isolated from all groups of mice (Fig. 6

the responses to sodium nitroprusside (SNP) were similar among

line (ACh), we did not observe detectable differences among any

of the groups with respect to vasodilation (Fig. 6C). Furthermore,

the responses to sodium nitroprusside (SNP) were similar among

vessels isolated from all groups of mice (Fig. 6D). This suggests

strongly that vascular smooth muscle function in both WT and

TLR2KO is unimpaired by HFD. Thus, consistent with our in

vitro experiments in cultured endothelial cells, these ex vivo

results suggest that HFD inhibits insulin-stimulated vasodilator

actions through a TLR2-mediated mechanism. This may help explain in part how HFD contributes to impaired metabolic homeostasis and cardiovascular function.

DISCUSSION

In the present study, we found that TLR2 plays an important role in SFA-induced inflammation and subsequent impairment of vasodilator actions of insulin. This is the first report demonstrating that TLR2 mediates HFD/SFA-induced impairment of insulin signaling and action in vascular endothelium related to endothelial dysfunction. Interestingly, SFA stimulates UPR by increasing splicing of XBP-1 to mature XBP-1s, leading to increased production of TNFα. Because TNFα causes endothelial dysfunction (37, 59), our findings suggest that SFA-mediated impairment of vascular actions of insulin may be due in part to TLR2/UPR/proinflammatory signaling and action.

Our results yield several novel insights into SFA-stimulated inflammatory responses in endothelium. First, TLR2-mediated inflammatory responses are composed of both UPR-dependent and UPR-independent mechanisms (Fig. 7). Second, impaired insulin signaling and action in vascular endothelium mediated by TLR2 may contribute to endothelial dysfunction induced by HFD and/or SFA that contributes to cardiovascular complications. Third, TLR2-mediated impairment of insulin signaling and action in the vasculature may help link dysregulation of metabolic homeostasis with cardiovascular complications of diabetes, metabolic syndrome, and obesity.

Link between insulin resistance and impairment of vascular actions of insulin. Obesity induces insulin resistance and endothelial dysfunction (18). The link between metabolic and vascular actions of insulin is determined in part by insulin-stimulated capillary recruitment that is responsible for ~40% of insulin-stimulated glucose uptake in skeletal muscle (2, 23). We show that TLR2 mediates HFD-induced impairment of vascular insulin action (Fig. 6B). The insulin-stimulated capillary recruitment is NO dependent and activated through a canonical PI 3-kinase-dependent insulin signaling pathway whose components include insulin receptor/IRS-1/PI 3-kinase/PDK/Akt/eNOS (Fig. 7) (31, 57, 58). Impairment of insulin-stimulated eNOS activity contributes to reduced blood flow that helps determine metabolic insulin resistance and glucose intolerance (18, 52). Indeed, complete loss of insulin signaling in the vascular endothelium as a result of tissue-specific knock-out of the insulin receptor promotes atherogenesis and increases expression of cell adhesion molecules (42). Thus, insulin signaling in vascular endothelium has specific effects to oppose inflammation and contribute to hemodynamic homeostasis. To complement our in vivo data with TLR2KO mice, our in vitro cellular studies and ex vivo data also demonstrate that TLR2 plays an important role in SFA-mediated impairment of vascular insulin action. Furthermore, we observed that there is a difference in insulin-stimulated vasodilation in WT chow vs. WT HFD (P < 0.05) but not in TLR2KO chow vs. TLR2KO HFD (P > 0.05) at 100 nM insulin (Fig. 6). These results are consistent with the results from in vitro cell culture studies. Thus, TLR2-mediated vascular insulin resistance may contribute to both metabolic and cardiovascular pathophysiology.

WT mice on HFD have impaired insulin-stimulated vasodilation compared with the same mice on a normal chow diet (Fig. 6B). By contrast, there is no detectable difference in ACh-stimulated vasodilation among these groups (Fig. 6C). Insulin and ACh stimulate production of NO by distinct mechanisms. Insulin stimu-

Fig. 6. Absence of TLR2 protects against HF diet induced impairment of vascular actions of insulin. C57BL/6J and TLR2−/− mice were fed with control chow or HF diet for 10 wk. Mesenteric arteries were isolated from mice. Arteries were preconstricted with phenylephrine (1 μM) and then exposed to increasing concentrations of insulin (A and B), acetylcholine (C), or sodium nitroprusside (SNP; D). The vasodilator actions in response to each agonist were observed and recorded using a data acquisition system. In some experiments (A), Nω-nitro-l-arginine methyl ester (l-NAME; 100 μM, 30 min) was applied prior to insulin treatment. A: mesenteric arteries were dilated in response to insulin in a concentration-dependent manner, and this was substantially blocked by pretreatment of l-NAME. B: values that are significantly different between C57BL/6J HF vs. TLR2−/− HF are indicated. Insulin-stimulated vasodilation was impaired in WT mice but not in TLR2−/− mice fed HF diet when compared with mice fed normal chow diet. C and D: Vasodilator responses to acetylcholine or SNP were not significantly different among any of the groups (C57BL/6J vs TLR2−/− without and with HF diet). The percentage of vasorelaxation was calculated as described in MATERIALS AND METHODS and presented as means ± SE. The data were analyzed by 2-way ANOVA for repeated measurements along with a Bonferroni post hoc test (evaluating differences between entire curves). *P < 0.05; ***P < 0.001.
TLR2 MEDIATES VASCULAR INSULIN RESISTANCE

Fig. 7. Schematic diagram of proposed TLR signaling pathways that inhibit insulin-stimulated vasodilation in vascular endothelial cells. Saturated FAs stimulate TLRs that transmit signaling to activate various Ser/Thr kinases, including IL-1β receptor-associated kinase 1, PKC, JNK, and IKKβ. These Ser/Thr kinases phosphorylate IRS-1 and -2 at serine residues, including Ser24, to inhibit insulin signaling through IR/IRS-1/phosphatidylinositol (PI) 3-kinase/Akt/eNOS. TLR signaling pathways stimulate UPR to promote maturation of XBP-1 to XBP-1s that stimulates TNFα production. In addition, TLRs stimulate UPR-independent pathways, including IKKβ/NF-κB, to stimulate production of IL-6 and E-selectin.

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Mechanisms for TLR-mediated insulin resistance. TLR-mediated inflammation contributes to insulin resistance in various tissues, including liver, adipose tissue, and vascular tissue (8, 10, 16, 24, 35, 48, 54). In the present study, we show for the first time that functional consequences of TLR2 activation to impair vascular actions of insulin may contribute to overall dysregulation of metabolic homeostasis. We observed that knockdown of TLR2 increased expression of TLR4 slightly (Fig. 1A). This may be a compensatory response for reduced expression of TLR2. However, even if this increased expression is “compensatory” in some contexts, it was clearly insufficient to substitute for the loss of TLR2 with respect to the vascular and metabolic functions that we investigated and did not have a detectable effect on the fatty acid-induced inflammatory responses that we evaluated. Our results are consistent with a previous study by Nguyen et al. (33) showing that knockdown of either TLR2 or TLR4 almost completely inhibited the fatty acid-induced responses. Inflammatory cytokines inhibit insulin signaling pathways in part through activation of serine/threonine kinases that phosphorylate negative regulatory S/T residues on insulin receptor substrates (36). We observed that Ser24 on IRS-1 is phosphorylated by SFA-stimulated TLR2 in primary endothelial cells (Fig. 4F). Previously, we reported that phosphorylation of IRS-1 at Ser24 inhibits insulin-stimulated IRS-1 binding and activation of PI 3-kinase and MAPK-dependent insulin signaling pathways may lead to increased secretion of endothelin-1 with a resultant increase in vasoconstrictor tone that augments the impaired endothelial dysfunction observed with vascular insulin resistance (39). Thus, it is possible that vasoconstrictor responses to ET-1 are augmented in HFD-induced vascular insulin resistance and that TLRs may be involved in these vascular actions of insulin. We did not address this possibility directly in the present work. However, these additional considerations merit further investigation in our future studies.

TLRs are involved in innate immunity and HFD-induced insulin resistance (4, 48). Deficiency of TLR2 does not protect from HFD-induced obesity (Fig. 5A), but it does protect from metabolic insulin resistance (Fig. 5E) and impaired insulin signaling and action in the vasculature (Figs. 4 and 6). These results suggest that SFA directly impairs insulin signaling and action in vascular endothelial cells, contributing to endothelial dysfunction. Moreover, lack of TLR2 in vascular endothelium is sufficient to uncouple obesity from both metabolic and vascular insulin resistance. Nevertheless, we cannot exclude the possibility that the inhibition of insulin actions by HFD or SFA under pathophysiological conditions may be due at least in part to other direct or indirect effects of hyperglycemia and hyperinsulinemia/insulin resistance. Cross-talk between metabolic and vascular tissues underlies reciprocal relationships between insulin resistance and endothelial dysfunction that compound deleterious effects of HFD on metabolic and cardiovascular function. Tissue-specific knock-out of TLR2 in the vasculature in our study opposes this pathophysiology. Lack of TLR was able to protect against HFD-induced obesity in some (24, 38), but not all (8, 48), previous studies. From these mixed results, it remains unclear whether TLRs play a role in HFD-induced body weight gain. Differences in these results among these studies may be due to differences in the background mice strain, components of diet, the duration of diet, or gut microbiota (5).
GLUT4 translocation in adipose cells (12, 19). Ser\textsuperscript{24} is located in the pleckstrin homology domain in IRS-1 that contributes to binding of IRS-1 to phospholipids (19). Thus, phosphorylation of IRS-1 at Ser\textsuperscript{24} inhibits interaction of IRS-1 with phospholipids, including the plasma membrane. The known protein kinases responsible for phosphorylation of IRS-1 at Ser\textsuperscript{24} are IL-1β receptor-associated kinase 1 and PKCs (12, 19) that are downstream components of TLRs (1, 7, 25, 49). Production of other cytokines by TLR-mediated mechanism may potentiate this negative regulation of insulin signaling pathways. However, more detailed mechanisms for inhibition of insulin signaling by TLRs remain to be elucidated.

TLR-mediated ER stress and apoptosis have been observed in atherosclerosis (46, 53). However, mechanisms linking ER stress and inflammation are not well understood. Our study suggests, for the first time, that the UPR plays a role in SFA-induced proinflammatory response through a TLR2-mediated mechanism (Fig. 3). XBP-1s plays a role in the production of TNFα that in turn contributes to endothelial dysfunction and insulin resistance (14, 55, 59). Thus, increased production of TNFα may contribute importantly to the impairment of endothelial vasodilator properties.

We conclude that SFA-induced impairment of vasodilator actions of insulin is mediated in part through TLR2. TLR2-mediated UPR and proinflammatory responses may represent mechanisms that contribute to vascular insulin resistance. Thus, our study has uncovered an additional potential mechanism for SFA-induced impairment of vascular function. This may contribute importantly to the pathophysiology of diabetes, metabolic syndrome, obesity, and their cardiovascular complications.

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DISCLOSURES

M. J. Quon is a member of the Merck Speaker Bureau.

AUTHOR CONTRIBUTIONS


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