Toll-Like Receptor-4 Mediates Vascular Inflammation and Insulin Resistance in Diet-Induced Obesity

Francis Kim, Matilda Pham, Ian Luttrell, Douglas D. Bannerman, Joan Tupper, Joshua Thaler, Thomas R. Hawn, Elaine W. Raines, Michael W. Schwartz

Abstract—Vascular dysfunction is a major complication of metabolic disorders such as diabetes and obesity. The current studies were undertaken to determine whether inflammatory responses are activated in the vasculature of mice with diet-induced obesity, and if so, whether Toll-Like Receptor-4 (TLR4), a key mediator of innate immunity, contributes to these responses. Mice lacking TLR4 (TLR4−/−) and wild-type (WT) controls were fed either a low fat (LF) control diet or a diet high in saturated fat (HF) for 8 weeks. In response to HF feeding, both genotypes displayed similar increases of body weight, body fat content, and serum insulin and free fatty acid (FFA) levels compared with mice on a LF diet. In lysates of thoracic aorta from WT mice maintained on a HF diet, markers of vascular inflammation both upstream (IKKβ activity) and downstream of the transcriptional regulator, NF-κB (ICAM protein and IL-6 mRNA expression), were increased and this effect was associated with cellular insulin resistance and impaired insulin stimulation of eNOS. In contrast, vascular inflammation and impaired insulin responsiveness were not evident in aortic samples taken from TLR4−/− mice fed the same HF diet, despite comparable increases of body fat mass. Incubation of either aortic explants from WT mice or cultured human microvascular endothelial cells with the saturated FFA, palmitate (100 μmol/L), similarly activated IKKβ, inhibited insulin signal transduction and blocked insulin-stimulated NO production. Each of these effects was subsequently shown to be dependent on both TLR4 and NF-κB activation. These findings identify the TLR4 signaling pathway as a key mediator of the deleterious effects of palmitate on endothelial NO signaling, and are the first to document a key role for TLR4 in the mechanism whereby diet-induced obesity induces vascular inflammation and insulin resistance. (Circ Res. 2007;100:1589-1596.)

Key Words: nitric oxide ■ Toll-like receptor-4 ■ free fatty acids ■ obesity

In conditions of nutrient excess such as obesity and diabetes, elevated free fatty acid (FFA) levels are implicated in the pathogenesis of both inflammation and insulin resistance in a variety of tissues, including endothelial cells.1–4 At the cellular level, nutrient excess is linked to insulin resistance via activation of IKKβ and, subsequently, NF-κB, a key transcriptional mediator of inflammation.5–6 In peripheral insulin-sensitive tissues such as muscle and liver, the pattern recognition receptor, Toll-like receptor-4 (TLR4) is implicated as a mediator of this effect.7 The current work was undertaken to determine whether obesity induced by high-fat (HF) feeding causes inflammation and insulin resistance in vascular tissue in vivo, and whether TLR4 contributes to this effect.

The bacterial endotoxin, lipopolysaccharide (LPS), is a potent activator of IKKβ and NF-κB in most cell types. The majority of the biological activity of LPS is contained within a moiety ("lipid A") that is acylated with saturated fatty acids, and removal of these fatty acids results in complete loss of endotoxic activity.8,9 Recently, TLR4 was shown to be required not only for LPS-induced inflammatory responses, but for responses to nonbacterial ligands such as lauric acid (C 12:0), a saturated fatty acid.10,11 These in vitro studies suggest that activation of TLR4 by certain FFA species can trigger cellular inflammatory responses. Whether TLR4 signaling contributes to the link between nutrient excess, inflammation, and metabolic dysfunction in vivo is an important unanswered question.

TLR4 is expressed on virtually all human cells and binds a wide spectrum of exogenous and endogenous ligands (including bacterial LPS) and is involved in innate immune responses to various infectious agents and stressors.12,13 In the presence of LPS, the TLR4 receptor complex (which includes CD-14 and an accessory protein, MD-2), recruits the adapter protein, myeloid differentiation factor-88 (MyD88). MyD88 in turn recruits interleukin-1 receptor–associated kinase (IRAK) and, by activating IKKβ and NF-κB, ultimately induces the expression of numerous inflammatory mediators. Recent evidence suggests that inflammatory processes induced by nutrient excess cause systemic insulin resistance via...
a mechanism involving TLR4, and a similar mechanism may also contribute to atherogenesis, as genetic deficiency of either TLR4 or MyD88 confers protection against atherosclerosis. Based on evidence that TLR4 can be activated by saturated FFAs, that TLR4 is required for LPS-mediated activation of NF-κB in endothelial cells, and that palmitic acid, a saturated FFA, causes endothelial insulin resistance via a mechanism dependent on activation of IKKβ, we sought to determine (1) whether the TLR4/MyD88 complex mediates the deleterious effects of fat excess on vascular tissue and, if so, (2) whether IKKβ and NF-κB are downstream mediators of these responses.

We demonstrate that in samples of thoracic aorta from mice made obese through consumption of a HF diet, IKKβ activity and subsequent NF-κB–dependent induction of IL-6 mRNA and ICAM protein expression are increased and that these responses are associated with cellular insulin resistance and impaired insulin-stimulated phosphorylation of eNOS (peNOS). Despite comparable increases of body weight and fat content, however, vascular inflammation and impaired insulin responsiveness were not evident in aortic samples taken from TLR4−/− mice fed the same HF diet. To determine whether TLR4 signaling also mediates deleterious effects of FFAs in endothelial cells, we incubated human microvascular endothelial cells (HMECs) in the presence of palmitate. We found that palmitate-mediated activation of IKKβ was associated with NF-κB transcriptional activation, as judged by induction of inflammatory markers ICAM and IL-6, and that these responses and the subsequent induction of insulin resistance in HMECs can be prevented either by decreasing TLR4 expression or by inhibiting MyD88 or IRAK signaling. Because inhibition of NF-κB signaling also blocked the inhibitory effects of palmitate on endothelial insulin signaling and NO production, NF-κB is strongly implicated as a mediator of palmitate-induced endothelial dysfunction downstream of TLR4. These findings implicate the TLR4/MyD88 pathway in the deleterious effects of palmitate on endothelial insulin signaling and NO production, and are the first to document a key role for TLR4 in the mechanism whereby diet-induced obesity induces vascular inflammation and insulin resistance.

### Materials and Methods

An expanded Materials and Methods section containing details for cell culture studies, Western blotting, siRNA transfection, NO measurement, ex vivo aortic studies, mouse studies, and analysis can be found in the online data supplement available at http://circres.ahajournals.org.

### Animals

TLR4−/− mice were kindly provided by Dr S. Akira (Osaka University, Osaka, Japan) and were backcrossed to the C57Bl/6 background (more than 9 generations) before study. Wild-type (WT) C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

### Results

#### Inflammation and Insulin Sensitivity of Aortic Tissue From WT and TLR4−/− Mice Fed a HF Diet

This study was undertaken to determine whether (diet-induced obesity, DIO) induced by a HF diet causes vascular inflammation and impaired endothelial insulin signaling in vivo and if so, whether TLR4 signaling is required for this effect. Compared with mice of either genotype fed a low-fat (LF) diet, HF feeding for 8 weeks increased body weight, body fat content, and serum levels of insulin, FFA, and cholesterol (supplemental Figure IA through IG). TLR4 deficiency had no detectable effect on body adiposity or humoral responses measured during consumption of either diet, with the exceptions of elevated serum FFA and triglycerides levels, which achieved statistical significance in TLR4−/− but not in WT mice.

To determine whether HF diet–induced obesity activates inflammatory signaling in vascular tissue in vivo, we measured IKKβ activity, as determined by the phosphorylation of IκBα in lysates of thoracic aorta removed from animals in each of the 4 groups (WT or TLR4−/− mice fed a LF or HF diet). HF feeding for 8 weeks induced a significant 2-fold increase of IKKβ activity in aortic tissue taken from WT mice compared with LF-fed controls (Figure 1A). In TLR4−/− mice maintained on a HF diet, however, no such increase of IKKβ activity was detected. Among many inflammatory genes induced by NF-κB are IL-6 and ICAM1. To confirm that NF-κB was indeed activated in thoracic aortic tissue exhibiting increased phospho-IκBα, we determined whether exposure to HF feeding increased IL-6 or ICAM expression, and whether absence of TLR4 signaling blocks this effect. As predicted, HF feeding was associated with increases of aortic ICAM1 and IL-6 mRNA levels in WT mice, but not in TLR4−/− mice (Figure 1B and 1C). The induction of inflammatory genes was not attributable to atherosclerosis lesion formation in C57Bl/6 mice on a HF diet because previous reports show that these mice do not demonstrate evidence of atherosclerosis in the aorta, even after 40 weeks on a HF diet.

To assess the effect of HF feeding on aortic insulin signaling, a subset of mice from each group received IP injections of either vehicle or insulin (2 U in 300 μL of normal saline) and 15 minutes later, thoracic aorta was removed and aortic lysates analyzed for pAkt and peNOS activity. As expected, insulin-stimulated pAkt and peNOS activation were impaired in aortic tissue from WT mice fed the HF diet compared with LF-fed controls (Figure 1D and 1E). As in other insulin sensitive tissues, therefore, biochemical responses to insulin are impaired in arterial tissue taken from WT mice with DIO, including phosphorylation of eNOS serine 1177 (peNOS), an endothelial-specific measure. In contrast, HF feeding did not attenuate insulin-induced activation of Akt or eNOS in aortic tissue taken from TLR4−/− mice. Collectively, these findings indicate that DIO induced by HF feeding promotes vascular inflammation and insulin resistance in vascular tissue via a mechanism that requires intact TLR4 signaling. Similarly, we found that insulin stimulation of pAkt in liver lysates was impaired by HF feeding in WT mice, but not in mice lacking TLR4 (supplemental Figure II), consistent with a recent report.

#### Effect of Palmitate on Vascular Tissue Treated Ex Vivo

As a first step to delineate mechanisms linking DIO to IKKβ activation and impaired insulin signaling in aortic tissue, we...
developed an ex vivo incubation assay system to determine whether brief exposure of aortic explants to palmitate, an abundant FFA in serum, mimics the deleterious effects of HF feeding. Using thoracic aorta taken from nonobese WT C57Bl/6 mice fed a standard chow diet, we found that ex vivo feeding. Using thoracic aorta taken from nonobese WT C57Bl/6 mice fed a standard chow diet, we found that ex vivo incubation with palmitate-BSA (100 μmol/L) for 3 hours increased IκBα phosphorylation (a measure of IKKβ activity) by >5-fold compared with BSA alone (Figure 2A). Because aortic tissue samples contain many different cell types that might contribute to this inflammatory response, we next sought to determine whether palmitate exposure specifically impairs endothelial function in this assay. In support of this hypothesis, ex vivo insulin stimulation (100 nmol/L for 15 minutes) of thoracic aortic tissue taken from WT mice increased phosphorylation of eNOS serine 1177 (a response that is specific to endothelial cells, as other cell types do not express eNOS), and this effect was blocked by pretreatment with palmitate-BSA for 3 hours in the absence of any change of eNOS protein level.

To determine whether these effects of palmitate are dependent on TLR4 signaling, we repeated these experiments using aortic samples obtained from chow-fed TLR4−/− mice. Unlike the evidence of cellular inflammation and insulin resistance seen in vascular tissue from WT mice after incubation in palmitate for 3 hours, this intervention did not activate IKKβ, nor did it impair insulin-stimulated activation of peNOS (Figure 2A and 2B) in aortic tissue from TLR4-deficient mice. Thus, TLR4 signaling is required for vascular inflammation and insulin resistance induced by either chronic exposure to a HF diet or brief ex vivo exposure to palmitate.

Role of TLR4 in the Response of Cultured Endothelial Cells to Palmitate

To determine whether TLR4 signaling mediates palmitate-induced inflammation and insulin resistance in endothelial cells, we transfected HMECs with either TLR4 siRNA to decrease TLR4 expression or with scrambled siRNA as a
control. The reduction of TLR4 expression after transduction with siRNA to TLR4 was confirmed by Western blot with an anti-TLR4 antibody (Figure 3A). Transduced HMECs were treated with palmitate-BSA (100 μmol/L for 3 hours) or with either of 2 other known activators of IKK—TNF-α (5 ng/mL for 1 hour), which does not signal via TLR4, or LPS (10 ng/mL for 1 hour), for which IKK activation is TLR4-dependent (Figure 3B). In control HMECs (transduced with scrambled siRNA), palmitate, TNF-α, and LPS each activated IKKβ, as assessed by increased IκBα phosphorylation. As expected, treatment with TLR4 siRNA blocked IKKβ activation in response to LPS, whereas the response to TNF-α remained intact despite reduced TLR4 signaling. Importantly, the ability of palmitate to activate IKKβ was also blocked by TLR4 siRNA, implicating TLR4 in the mechanism underlying IKKβ activation induced by palmitate and LPS, but not by TNF-α, in endothelial cells. Thus, IKKβ appears to be a downstream mediator of TLR4-mediated inflammatory responses induced by palmitate.

We next asked whether TLR4 is necessary for palmitate-mediated impairment of endothelial insulin signaling and eNOS activity. Consistent with earlier results, insulin-stimulated pAkt induction and NO production were inhibited in HMECs incubated with palmitate and scrambled control siRNA (Figure 3C). After treatment with TLR4 siRNA, however, these effects of palmitate were no longer detectable (Figure 3C and 3D), suggesting that TLR4 is necessary for palmitate-induced impairment of endothelial insulin signaling and NO production.

Role of NF-κB in Palmitate-Mediated Inhibition of NO Production in Endothelial Cells

We previously reported that IKKβ is both necessary and sufficient for the inhibitory effect of palmitate on insulin-induced NO production.1 One mechanism proposed to mediate this effect is that activation of IKKβ in turn activates NF-κB, and that this response initiates a cascade of events that ultimately inhibit insulin-mediated Insulin-Receptor Substrate-1 (IRS-1) tyrosine phosphorylation, Akt serine 473 phosphorylation (pAkt), eNOS serine 1177 phosphorylation (peNOS), and NO production.1,20 According to this hypothesis, NF-κB activation should be necessary for palmitate-induced inhibition of insulin-signaling and NO production in endothelial cells. To test this hypothesis, we used HMECs transduced with either a phosphorylation-resistant mutant of IκBα that blocks NF-κB (NF-κB super repressor)21 or control vector expressing GFP. Transduced HMECs were treated with palmitate-BSA (100 μmol/L for 3 hours), TNF-α (5 ng/mL for 1 hour), or LPS (10 ng/mL for 1 hour). In control GFP-transduced HMECs, palmitate, TNF, and LPS each increased phosphorylation of IκBα. In contrast, this response was not seen in HMECs transduced with the
IκBα-resistant mutant (Figure 4A), indicating that NF-κB was not activated. Consistent with earlier results, insulin-stimulated IRS-1 tyrosine phosphorylation, pAkt induction, and NO production were also inhibited by palmitate in control HMECs transduced with GFP (Figure 4B through 4D), whereas in HMECs transduced with NF-κB super repressor, these responses to palmitate were no longer detectable (Figure 4B through 4D). Collectively, these results suggest that NF-κB signaling acts downstream of TLR4 and is necessary for palmitate-induced impairment of endothelial insulin signaling and NO production.

**Role of MyD88 Signaling on Palmitate-Induced Endothelial Dysfunction**

MyD88, by virtue of its direct recruitment to TLR4, is a proximal signaling molecule involved in LPS-induced NF-κB activation. To determine whether MyD88 is also required for FFA-mediated activation of IKKβ, we used HMECs stably transfected with either a dominant negative (DN) mutant of MyD8822 or a control construct expressing GFP. After treatment with palmitate-BSA (100 μmol/L for 3 hours), TNF-α (5 ng/mL for 1 hour), or LPS (10 ng/mL for 1 hour; Figure 5A), GFP-transfected HMECs exhibited the expected increase of IκBα phosphorylation, indicating increased IKKβ activity. As predicted, HMECs transfected with DN-MyD88 displayed increased IKKβ activity in response to TNF-α, because TNF-α activates IKKβ via a mechanism that is not dependent on TLR4 or MyD88 signaling.23 In contrast, activation of IKKβ in response to either LPS or FFA was strongly attenuated by transfection with DN-MyD88. Furthermore, the ability of insulin (100 nmol/L insulin for 5 minutes) to increase IRS-1 tyrosine phosphorylation, Akt serine phosphorylation, and NO production in HMECs transfected with control vector was also attenuated by palmitate, and this inhibitory effect was prevented by transfection with DN-MyD88 (Figure 5B through 5D). These data collectively indicate that, like TLR4 and NF-κB, MyD88 is required for the deleterious effects of both palmitate and LPS on endothelial function.

**Role of IRAK in Endothelial Cell Responses to Palmitate**

IRAK-1 functions as an adapter protein within the TLR signaling complex. After receptor activation MyD88 binds to IRAK-1, which in turn activates IKKβ, and subsequently NF-κB, a step known to be essential for cellular inflammation induced by LPS. To investigate the role of IRAK-1 in palmitate-induced activation of IKKβ in endothelial cells, HMECs were stably transfected with a gene encoding a truncated mutant of IRAK-1 that interrupts LPS-induced NF-κB activation.22 As shown in Figure 5A, exposure to palmitate for 3 hours increased IKKβ activity in HMECs transduced with GFP, but not with the DN-IRAK mutant. Similarly, insulin-stimulated IRS-1 tyrosine phosphorylation, serine phosphorylation of Akt, and production of NO were inhibited by exposure to palmitate in control, but not in DN-IRAK-transfected, HMECs (Figure 5B through 5D).

To investigate whether NF-κB is activated downstream of TLR4, we determined whether exposure to inflammatory stimuli increases IL-6 or ICAM expression, and whether inhibition of TLR4 signaling blocks this effect. Toward this end, HMECs were incubated with palmitate, LPS, or TNF-α and levels of IL-6 and ICAM were determined. Each of these inflammatory stimuli induced both IL-6 and ICAM expression, and in the case of palmitate and LPS, but not TNF-α,

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Effect of inhibition of NF-κB signaling on Palmitate-mediated IκBα phosphorylation and endothelial insulin signaling. HMECs transduced with a phosphorylation resistant IκBα (NF-κB super repressor) or GFP construct were treated with palmitate (100 μmol/L), TNF-α (10 ng/mL), or LPS (10 ng/mL). A, Cell lysates were analyzed by Western blot analysis with an anti–phospho-IκBα antibody. B, Insulin mediated IRS-1 tyrosine phosphorylation C, Insulin-mediated phospho-Akt (ser 473) quantified from Western blots (3 independent experiments) of HMECs transduced with GFP or NF-κB super repressor. D, Insulin-mediated NO production as measured by cGMP assay. Fold increase over control (GFP-control) was calculated from 4 independent experiments. *P<.05.
these effects were inhibited by DN-MyD88 and DN-IRAK compared with GFP controls (supplemental Figure III). These results indicate that like LPS, palmitate activates NF-κB-mediated gene transcription through a TLR4-dependent pathway in endothelial cells.

**Discussion**

Growing evidence implicates the cellular inflammatory response to nutrient excess as a key mechanism linking cardiovascular disease to obesity and related metabolic disorders. A steady increase in the prevalence of these disorders has heightened the need for an improved understanding of how nutrient excess affects the vasculature. Toward this end, the current work was undertaken to determine whether HF feeding induces vascular inflammation and insulin resistance in vivo, and to ascertain the role played by TLR4 in these effects. First, we replicated the observation that in WT mice, obesity induced by HF feeding is associated with elevated circulating levels of both insulin and FFA, consistent with the development of insulin resistance. In this setting, our finding of IKKα activation and impaired insulin signaling in aortic tissue from WT mice fed a HF diet suggests that DIO causes inflammation and insulin resistance within the vasculature as well as in conventional insulin-sensitive tissues such as muscle, liver, and fat. Moreover, our finding that these vascular consequences of DIO did not occur in mice lacking TLR4, despite comparable increases of body weight and body fat mass, identifies TLR4 as a key mediator of vascular inflammation and insulin resistance in this setting.

The hypothesis that TLR4 plays a key role to impair vascular insulin signaling in mice with DIO is consistent with findings from recent studies. For example, FFAs were recently shown to activate TLR4 signaling in adipocytes and macrophages, and the ability of FFA to activate inflammatory signaling in these cells was blocked in the absence of TLR4. Moreover, DIO-induced systemic insulin resistance is attenuated in TLR4−/− mice and, in the current work, we found that insulin-mediated pAkt stimulation in liver is inhibited by DIO in WT mice, but not in mice lacking TLR4. Collectively, these findings implicate TLR4 as a key mediator of inflammation leading to insulin resistance under conditions of nutrient excess in many key tissues, including the vasculature.

To determine whether exposure to palmitate, an abundant FFA in mammalian plasma, can recapitulate the vascular inflammation and insulin resistance induced in WT mice by DIO, and to determine whether this effect involves TLR4, we incubated samples of aortic tissue from WT and TLR4−/− mice fed a LF diet in palmitate-containing media. As predicted, we found that whereas brief ex vivo incubation of aortic tissue from normal mice in palmitate-containing media increased IKKβ activity and reduced both insulin signaling and eNOS activity, these effects, like the response to HF feeding, were absent in vascular tissue from mice lacking TLR4. Deleterious effects of palmitate on isolated thoracic aorta, therefore, require intact TLR4 signaling.

A key question raised by these findings is whether TLR4-mediated vascular inflammation is mediated directly through vascular cells, through an indirect mechanism involving macrophages or other inflammatory cells, or whether both mechanisms contribute. Although additional studies are needed to address this question, our data indicate that in endothelial cells, TLR4 signaling is a critical mediator of palmitate-induced IKKβ and NF-κB activation and subsequent decreases of insulin signaling and NO production.
The cellular mechanism responsible for palmitate-induced impairment of NO production appears to involve impaired activation of IRS-1, which in turn induces cellular insulin resistance. The IRS-1/pAkt/peNOS pathway is important for endothelial NO production because transfection with inhibitory mutants of IRS-1, PI3-kinase, or Akt prevent insulin-stimulated NO production. Furthermore, many of the metabolic abnormalities associated with diabetes and obesity, including elevated circulating TNF-α and FFA levels, impair the IRS-1/PI3-kinase signaling pathway in a variety of cell types, and we have shown that exposing endothelial cells to TNF-α impairs insulin- and fluid shear-dependent NO production. Interestingly, excess glucose also impairs NO production through its inhibition of the IRS-1/pAkt/peNOS pathway in much the same way as shown for palmitate and TNF-α. Conversely, NO signaling in endothelial cells for which IRS-1 is not required, such as via VEGF-dependent activation of peNOS, is not impaired by palmitate (data not shown). These observations collectively suggest that inhibition of IRS-1/pAkt/peNOS signaling is an important mechanism whereby endothelial insulin signaling and NO production are impaired by nutrient excess.

In support of this hypothesis, we demonstrated that in cultured endothelial cells, reduced IRS-1 signaling and eNOS activation induced by palmitate are dependent on each of the key proteins in the TLR4 signaling pathway: TLR4, MyD88, IRAK, IKKβ, and NF-κB. These results constitute strong direct support for a model in which palmitate activates TLR4, which in turn engages MyD88 and IRAK, subsequently activating IKKβ and NF-κB. Activation of NF-κB then inhibits IRS-1 tyrosine phosphorylation via an as yet unidentified mechanism. Among several candidate mediators of NF-κB–mediated inhibition of IRS-1 signaling are S6-kinase-1, mTOR (mammalian target of rapamycin), suppressor of cytokine signaling (SOCS3), c-JUN-NH2 (JNK), Akt, and several PKC isoforms. Each of these enzymes share in common the ability to inhibit IRS-1 signaling by phosphorylating one or more of its 70 IRS-1 serine residues, an IRS-1 modification known to impair IRS-1 signaling.

Based on these collective results, TLR4 is strongly implicated in the development of endothelial inflammation, insulin resistance, and impaired NO production in lipotoxic conditions of FFA excess. Whether other cell types within the vasculature display similar responses in states of nutrient excess, and whether such effects are also dependent on TLR4 signaling, await further study. In this context, we note that our results do not exclude alternative mechanisms whereby TLR4 signaling may impair NO production. Among these is the possibility that reactive oxygen species are generated via the association between TLR4 and NADPH oxidase, and that these highly reactive molecules impair eNOS activity. Along with oleic and stearic acid, palmitic acid is 1 of 3 FFA species that constitute ~70% of the total circulating FFA pool and is normally present in concentrations between 10 to 50 μmol/L. Our cell culture and ex vivo studies used palmitic acid complexed with BSA at a concentration of 100 μmol/L, slightly higher than is usually found in plasma. In other studies, however, we found that IKKβ is activated in endothelial cells by palmitate/BSA at concentrations as low as 10 μmol/L, which supports the physiological relevance of our findings and raises the interesting but untested possibility that endothelial cell insulin sensitivity and nitric oxide production are constrained by FFA at circulating levels that are within the physiological range. Combined with our current results, we hypothesize that TLR4 signaling plays a key role to mediate vascular responses to FFAs.

If prolonged, vascular inflammation and endothelial dysfunction may contribute to atherosclerosis in the setting of obesity. A role for TLR4 in the pathogenesis of atherosclerosis is suggested by evidence that TLR4 is present both in human atherosclerotic plaque and in murine models of atherosclerosis, and that expression of this receptor in both endothelial cells and macrophages is upregulated by oxidized LDL, a key proinflammatory agent present during the development of atherosclerosis. Furthermore, deficiency of either TLR4 or MyD88 attenuates diet-induced atherosclerosis in apolipoprotein E−/− mice. Although TLR4 likely contributes to atherosclerosis progression via effects in macrophages and other inflammatory cells, results of the present study suggest that consumption of a diet high in saturated fats can induce vascular inflammation and insulin resistance through activation of local TLR4 signaling in endothelial and perhaps in other vascular cell types, and that these responses can impair nitric oxide production and favor atherosclerosis progression. Based on these collective observations, we conclude that the TLR4/MyD88 pathway is a key mediator in the mechanism linking DIO with vascular dysfunction.

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Disclosures
None.

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Supplementary Information

Methods

Materials: Anti-phospho-eNOS (Ser1177), anti-TLR4, phospho-Akt (Ser473), anti-Akt, anti-phospho-IκBα rabbit polyclonal antibodies and anti-phosphotyrosine monoclonal antibody were obtained from Cell Signaling (Beverly, MA), anti-eNOS antibody was obtained from Transduction Labs, BD Biosciences (Lexington, Kentucky), and anti-human ICAM-1 from R &D Systems, Inc (Minneapolis, MN). Total Akt and pAkt(serine 473) ELISA kits were obtained from Biosource (Camarillo, CA) and Human IL-6 ELISA kit from R&D Systems. Regular human insulin (Humulin) was purchased from Eli Lilly Inc. (Indianapolis, IN). RPMI was purchased from Clonetics (Walkersville, MD) and Dulbecco’s Modified Eagle’s Medium (DMEM) was obtained from Biowhittaker (Rockland, ME). Palmitic (C 16:0) and oleic (C 18:1) fatty acids were obtained from Alltech Associates, Inc. (Deerfield, Illinois). BSA (FFA-free) was purchased from Roche (Indianapolis, In). FFA were dissolved in 0.1 M NaOH at 70°C and then complexed with 10% BSA at 55°C for 10 min for a final palmitate concentration of 100 µM as described previously18. Stock solutions of 5 mM FFA with 10% BSA and 10%BSA control solutions were prepared one day prior to experiments, and were added to HMEC. Palmitate preparations were assessed for LPS contamination using Amebocyte Lysate Test (Biowhittaker). Recombinant TNF-α was purchased from R&D Systems (Minneapolis, Minn) and LPS was purchased from Sigma Chemical Company (St. Louis, MO).

Animals: TLR4-/- mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan) and were backcrossed to the C57Bl/6 background (greater than 9 generations) prior to study. Wild-type (WT) C57Bl/6 mice were purchased from Jackson Laboratories. All animals were maintained in a temperature-controlled facility with a 12-hour light-dark cycle. Age-matched male C57Bl/6 mice (n=20) and TLR4-/- mice (n=20) (6-12 weeks old) were fed either low-fat (10% saturated fat)
or HF (60% saturated fat) diets that were otherwise matched for calories and micronutrients (Research Diets, numbers D12492, D12450B) for 8 weeks.

Total serum FFA levels were determined in the fed condition using enzymatic assay (Wako, Richmond, VA). Serum was also collected at the time of sacrifice and insulin levels were determined using an ELISA kit (Cyrstal Chem Inc, Downers Grove, IL), and glucose levels were determined by glucometer (FreeStyle, TheraSense, Alameda, CA).

**Cell Culture:** Human microvascular endothelial cells (HMEC, a generous gift from FJ Candal and T. Lawley, Emory University, Atlanta, GA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 12 μg/ml of bovine brain extract (Clonetics, Walkersville, MD), L-glutamine (2 mM), sodium pyruvate (1 mM) and nonessential amino acids in the presence of penicillin (100 units/ml) and maintained at 37°C in 5% CO₂. All Western blots were performed as described¹, using equal amounts of total protein for each condition and experiment. SDS gel electrophoresis was performed using a 4% by 20% gradient gel. Generation of stably-transfected HMEC with DN-MyD88 and DN-IRAK was as previously described.²⁴ The Silencer™ SiRNA Transfection Kit II (Ambion) was used to reduce expression TLR4. The SiRNA sequence generated to TLR4 5'-3' was: GGAACUUGGAAAAGUUUG and antisense: CAAACUUUUCCAAGUUUCC. Silencer™ Negative Control siRNA was used as the scrambled control.

**Total RNA extraction, quantitative RT-PCR**

Thoracic aortic tissue total RNA was extracted using RNeasy Mini Kit (Qiagen). Mouse IL-6 primer pairs were purchased from Applied Biosystems.
NO measurement: Intracellular cGMP levels reflect NO bioactivity and were measured using a commercially available cGMP immunoassay kit (Cayman Chemical Co, Ann Arbor, MI).

Mice were weighed and food intake measured weekly. Baseline, 4 wk, and 8 wk body composition measurements were assessed non-invasively using NMR spectrometry (Echo Medical Systems)\(^1\) in the University of Washington Clinical Nutrition Research Unit Body Composition and Energy Expenditure Core Laboratory.

At study conclusion, each animal received an IP injection of vehicle (normal saline) or regular insulin (2 U in 300 µl of normal saline) after an overnight fast. Fifteen minutes later, mice were anesthetized with isoflurane and the thoracic aorta was removed and surrounding connective tissue was quickly removed and the aortic tissue was snap frozen. Protein was extracted from aortic tissue samples and, after protein levels were quantified using Micro BCA Protein Assay Kit (Pierce, Rockford Il), equal amounts of protein were used for each condition in each assay. Total Akt and phospho-Akt (serine 473) levels were determined using ELISA assay kits (Biosource). Total eNOS, peNOS, and phospho-\(\kappa\)B\(\alpha\) were assessed using Western blot analysis and were quantified using Image J software (NIH). All procedures were approved by the University of Washington Animal Care and Use Committee.

**Ex-vivo aortic studies:** Mouse thoracic aorta were isolated from both wild-type (c57Bl/6) and TLR4/- mice following anesthesia with isoflurane. Aortic tissue was cleaned and placed into EBM media for 60 mins. Palmitate-BSA or BSA alone was then added to give a final concentration of 100 µM for 3 h. Aortic segments were then treated with insulin 100 nM or vehicle for 15 min.
Proteins were extracted and quantified and Western blot analysis was performed using an anti-phospho-IκBα, anti-p-eNOS and total eNOS antibodies.

**Statistical analysis:** In all experiments, densitometry measurements were normalized to controls incubated with vehicle and fold increase above the control condition was calculated. Analysis of the results was performed using the STATA8 statistical package. Values of p<0.05 were considered statistically significant. A two-tailed t test was used to compare mean values in two-group comparisons. To compare responses across genotypes, data were analyzed by two-way analysis of variance, and the Bonferoni-post-hoc comparison test was used to compare mean values between groups.

**Supplementary Figures Legend**

**Supplementary Figure 1.** The effect of 8 week of HF feeding on C57Bl/6 and TLR4-/- mice on body weight, body adiposity, and serum insulin and FFA levels.  

A. Body weight over time in the wild-type C57Bl/6 mice fed either an equicaloric low fat or HF diet.  

B. TLR4-/- mice body weight over time in response to same diet as in A.  

C. Mean body adiposity (± SD, n=10 in each treatment group) in response to HF feeding in the wild-type and TLR4-/- mice as measured by NMR spectrometry.  

D. Mean serum insulin levels (± SD, n=10 in each treatment group).  

E. Mean serum FFA levels (± SEM, n=6).  

F-G. Mean serum cholesterol and triglyceride(± SD, n= 10 each treatment group. (*, p<0.05)
Supplementary Figure 2. The effect of 8 weeks of HF feeding on C57Bl/6 and TLR4 -/- mice on insulin-mediated pAKT in liver tissue. Mice were given an IP injection of PBS or 2 units of insulin and then sacrificed after 15 min. Liver tissue were removed and cell lysates were made. pAkt levels were measured by ELISA and fold increase over control was calculated. *p<0.05.

Supplementary Figure 3. Effect of dominant negative MyD88 and IRAK on FFA-mediated IL-6 production and ICAM-1 expression in endothelial cells. A. HMEC transduced with DN-MyD88, DN-IRAK, or GFP were treated with FFA (100 mM), TNF-α (10 ng/ml) or LPS (10 ng/ml). Cell lysates were analyzed in non-reduced condition with an anti-ICAM antibody (representative of 3 independent experiments). B. Cell culture media IL-6 levels as measured by ELISA. Fold increase over control (GFP-control) was calculated (C=control, F= FFA, T=TNF-α, L=LPS).

References


Supplementary Figure 1
Fold increase in pAkt with insulin

C57Bl6  TLR4 -/-
A

Supplementary Figure 3

B