Toll-like receptor 4-induced endoplasmic reticulum stress contributes to impairment of vasodilator action of insulin

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Short Title: TLR4 induces endothelial dysfunction by ER stress

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Key Words: Endothelial dysfunction, TLR4, ER stress, insulin, nitric oxide
ABSTRACT

Impairment of vasodilator action of insulin is associated with endothelial dysfunction and insulin resistance. Activation of toll-like receptor 4 (TLR4) induces pro-inflammatory response and ER stress. Saturated fatty acids (SFA) activate TLR4, which induces ER stress and endothelial dysfunction. Therefore, we determined whether TLR4-mediated ER stress is an obligatory step mediating SFA-induced endothelial dysfunction. Palmitate stimulated pro-inflammatory responses and ER stress, and this was suppressed by knock-down of TLR4 in primary human aortic endothelial cells (HAEC). Next, we examined the role of TLR4 in vasodilatory responses in intact vessels isolated from wild type (WT, C57BL/6) and TLR4-KO mice after feeding high fat (HFD) or normal chow diet (NCD) for 12 weeks. Arterioles isolated from HFD WT mice exhibited impaired insulin-stimulated vasodilation compared to the arterioles isolated from NCD WT mice. Deficiency of TLR4 was protective from HFD-induced impairment of insulin-stimulated vasodilation. There were no differences in acetylcholine (Ach)-, or sodium nitroprusside (SNP)-stimulated vasodilation between two groups. Furthermore, we examined whether ER stress is involved in SFA-induced impairment of vasodilator actions of insulin. Infusion of palmitate showed the impairment of vasodilatory response to insulin, which was ameliorated by co-infusion with taouroursodeoxycholic acid (TUDCA), an ER stress suppressor. Taken together, the results suggest that TLR4-induced ER stress may be an obligatory step mediating the SFA-mediated endothelial dysfunction.
INTRODUCTION

One important role of vascular endothelium is to regulate vascular tone through balancing the production of vasodilators and vasoconstrictors such as nitric oxide (NO), endothelial derived hyperpolarizing factors and endothelin-1 (ET-1) (6, 18, 27). The relationship between endothelial dysfunction and insulin resistance (27) underlie clustering of cardiovascular and metabolic diseases including hypertension, atherosclerosis, diabetes, and obesity (42). Endothelial dysfunction caused by reduced bioavailability of NO plays an important role in the pathogenesis of cardiometabolic syndrome (47, 55, 57). One of the vascular actions of insulin increases blood flow that facilitates delivery of nutrients and hormones (25, 56, 65, 66). Vasodilator actions of insulin contribute to glucose uptake in skeletal muscle (2, 30, 37). Endothelial production of NO in response to insulin stimulation is mediated by signaling pathways involving the insulin receptor (IR) / IRS-1 / PI 3-kinase / PDK-1 / Akt. Akt directly phosphorylates eNOS at Ser1177 (40, 41, 65). Phosphorylation of eNOS at Ser1177 stimulates enzymatic activity resulting in increased production of NO (9, 16). Insulin-stimulated vasodilation is diminished in insulin-resistant animals and humans, which promotes various cardiovascular diseases including hypertension, atherosclerosis, and coronary heart disease (42, 57, 62).

Palmitate (PA) is the most abundant saturated fatty acid in human bloodstream (7, 58). Elevated circulating fatty acids in human subjects with obesity and diabetes contribute to impaired vascular function, which is associated with metabolic and cardiovascular disorders (1, 7, 58). Obesity-induced inflammatory responses are mediated, in part, by toll-like receptors (TLRs) that are activated by saturated fatty acids (SFA) (12, 14, 24, 29, 32, 53). SFA-stimulated TLR4 (33) increases expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 as well as adhesion molecules including E-selectin and intercellular adhesion molecule (ICAM) (24, 44,
Furthermore, TLR4 deficient mice are protected from various cardiovascular disease including septic cardiomyopathy, ischemia/reperfusion injury, heart failure, toxic cardiomyopathy and cardiac hypertrophy (15). Increased expression of TLR4 is observed in atherosclerotic plaques (11, 38, 39). Moreover, TLR4 deficient mice are protected against obesity-induced insulin resistance when compared with wild type mice (44, 52, 53). Thus, TLR4 may be a key link between obesity and insulin resistant diseases including diabetes and its cardiovascular complications.

Endoplasmic reticulum (ER) stress has been implicated as a cause of inflammation and insulin resistance, diabetes, and cardiovascular diseases (17, 28, 46). Despite numerous studies regarding TLR4-mediated pro-inflammatory responses and insulin resistance, it is unknown whether TLR4-induced ER stress is a mechanism by which SFA impairs the vasodilator actions of insulin. In the present study, we investigated whether TLR4-induced ER stress is an obligatory step mediating the SFA-induced pro-inflammatory responses and the impairment of vasodilator actions of insulin.

MATERIAL AND METHODS

Materials

Anti-eIFα, p-eIFα, anti-pI-κBα anti-p-Akt, and anti-Akt were obtained from Cell signaling (Beverly, MA). Anti-IRS-1 antibody was from Millipore (Billerica, MA) and anti-p-JNK was obtained from Invitrogen (Carlsbad, CA), and anti-phosphotyrosine, anti-p-p65NF-κB, and anti-JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DsiRNA for human TLR4 and non-targeted 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 Animal Use and Care Committee at The University of Alabama at Birmingham. Wild type mice (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME). TLR4KO mice are a kind gift from Dr. Shizuo Akira and Dr. Osamu Takeuchi (19). All animals were maintained in a temperature-controlled facility with a 12-hour light-dark cycle. At 6 week of age, mice were fed either chow (7917 Harlan Diet, 11% calories from fat) or high fat diet (Test Diet 5SPQ, 54% calories from fat, Richmond, IN) for 10 weeks. Mice were fasted overnight and anesthetized with sodium pentobarbital (50 mg/kg) before euthanization.

**Cell Culture and Transfection**

*Endothelial cell culture* - Bovine aortic endothelial cells (BAECs) were maintained in F-12K media containing 5% fetal bovine serum (FBS), endothelial cell growth supplement (BD Biosciences 30 mg/L), heparin sulfate (50 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Human aortic endothelial cells (HAECs, Lonza, Walkersville, MD) in primary culture were grown in F-12K media containing EGM-2 single quote supplements (Lonza, Walkersville, MD). All experiments were conducted on HAECs and BAECs before their sixth passage. HAECs were transiently transfected with 100 nM of siRNA duplex oligonucleotides (non-targeted scrambled or TLR4 DsiRNA) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to 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 legends to figures. BAECs were transfected with lipofectamine with plus as manufacturer’s instruction. Cells were transiently transfected with TLR4-DN (33, 34) or pcDNA3.1 (vector only) as following. Cells were incubated with transfection complex for 4 hrs
in OPTI-MEM media, and then replaced with regular media containing growth factors and serum for 48 hrs.

*Stable Transfection* – TLR4-WT and MD2 were described previously (33, 34). HEK293 cells were transfected with TLR4-WT and MD2 and treated with geneticine 418 (50 μg/ml) for three weeks. Growing colonies were selected.

**Preparation of palmitate**

Preparation of palmitate was carried out as described by Mott *et al.* (63). Briefly, 10.5% bovine serum albumin (Fitzgerald, MA) was dissolved in 25mM HEPES/DMEM, and filtered (0.22 μM, Millipore). Sodium palmitate was heated to be dissolved in water, and rapidly added to warmed BSA solution. Then this BSA-conjugated palmitate was added to reach the proper concentration of pamitate. We used endotoxin-free reagents and we tested all the reagents we used including BSA, palmitate, media and reagent diluents. We checked endotoxin level of all the reagents we used in this study by Chromogenic Endotoxin Quantitation assay kit (Pierce). Lower than 25 pg/ml is undetectable.

**Measurement of NO production**

Production of NO was assessed using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA) (EMD Biosciences, Gibbstown, NJ) as described previously 1. Briefly, endothelial cells were grown to 95% confluence in 24 well black plate (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 DAF-2 DA (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.

**Functional assessment for isolated mesentery 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 CaCl, 1.2 mM MgSO4, 1 mM NaH2PO4, 5 mM glucose, 3 mol/L 3-(N-morpholino) propan sulfonic acid (MOPS) buffer, 2 mM pyruvate, 0.02 mM EDTA, 1% BSA, pH 7.4). The isolated arterioles were then cannulated with glass micropipettes with 10-0 monofilament suture, and mounted in a custom-designed tissue chamber (Living System Instrumentation, Burlington, VT, USA). 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 contraction was achieved (5 min). The vessels were subsequently stimulated with various stimulators ($10^{-11}$ - $10^{-5}$ mol/L, 3 min per concentration), including insulin, acetylcholine, or 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 Inc., Colorado, Springs, CO). In some experiments, palmitate (200 µM) was intraluminally infused into vessels with and without tauroursodeoxycholic acid
(TUDCA, 500 μM), and incubated for 4 hrs. Then, vessels were incubated with the indicated vasodilators in different concentrations as described above. The data are expressed as means ± SE. The vasodilator responses to insulin were calculated as percent relaxation from the phenylephrine constriction according to the following equation:

\[
\text{Relaxation} \% = \frac{(\text{ID}^{\text{treat}} - \text{ID}^{\text{PE}})}{(\text{ID}^{\text{w/oCa}^{++}} - \text{ID}^{\text{PE}})} \times 100
\]

Where \( \text{ID}^{\text{treat}} \) 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 penylephrine; and \( \text{ID}^{\text{w/oCa}^{++}} \) 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 Na₃VO₄, 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 x 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 figures using standard methods. Immunoblots were visualized with quantified by Image analyzer (Vision Works LS) and UVP.

**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 Omniscript RT Kit (Qiagen,
Valencia, CA). Then the cDNA was subjected to semi-quantitative PCR analysis by using Hot Star Taq Master Mix kit (Qiagen, Valencia, CA). PCR product was visualized with fluorescent dye (Envirosafe DNA/RNA Stain, Helixx Technologies, Inc, Ontario, Canada) and the image was analyzed and quantified by Image analyzer (Vision Works LS) and UVP. The primers for each gene are described in the Table 1.

**Statistical Analysis**

Values are presented as mean ± standard error of the mean (SEM). Western blots were analyzed by one-way ANOVA. For the comparisons of vasodilatory responses, repeated measurements of two-way ANOVA was used.

**RESULTS**

*Palmitate stimulates pro-inflammatory responses through a TLR4-dependent mechanism* – To determine whether palmitate stimulates inflammatory responses through TLR4 in vascular endothelial cells, cells were transiently transfected with siRNA for TLR4 or scrambled, and treated HAEC with BSA or palmitate. Next, we examined the expressions of pro-inflammatory genes. Transfection of siRNA for TLR4 reduced the expression of TLR4, but the expression of TLR2 was not altered. Treatment with palmitate increased the expression of pro-inflammatory genes including *E-selectin, TNF-α, IL-1β* and *IL-6*, but not *MCP-1*, and knock-down of TLR4 abolished the palmitate-induced inflammatory responses (Fig. 1).

*Palmitate stimulates pro-inflammatory signaling molecules through a TLR4-dependent mechanism* – Next, we examined whether palmitate stimulates pro-inflammatory signaling molecules. Phosphorylations of JNK and IκBα were evaluated by immunoblottings. Treatment with palmitate stimulated phosphorylation of JNK and IκBα, while knock-down of TLR4 blunted the effects of palmitate to stimulate phosphorylation of JNK and pIκBα. (Fig. 2).
**Palmitate stimulates ER stress through a TLR4-dependent mechanism** – Metabolic stress causes ER stress that leads to inflammatory responses (20). We examined whether TLR4 contributes to palmitate-induced ER stress. Treatment with palmitate stimulated splicing of XBP-1 in cells transfected with scrambled siRNA, while splicing of XBP-1 was not increased in TLR4-knocked-down endothelial cells (Fig. 3A). By contrast, the expressions of other ER stress markers including GRP78 and CHOP were not altered by treatment with palmitate (Fig. 3A). As a cellular protective mechanism, translation of proteins is inhibited when cells undergo ER stress. Phosphorylation of eIF2α is one of the markers for ER stress. Treatment with palmitate increased phosphorylation of eIF2α, and knock-down of TLR4 blunted the palmitate-induced phosphorylation of eIF2α (Fig. 3B).

**Activation of TLR4 impairs insulin signaling** – There have been studies suggesting stimulation of TLR4 is a cause of insulin resistance (49, 50, 53, 60). To confirm that SFA-induced insulin resistance is mediated through TLR4 signaling pathways, we stably overexpressed TLR4 with MD2 in HEK293, which does not express TLR4. TLR4 overexpressing HEK293 cells were treated with palmitate, Pam3CSK4, or LPS, and then the cells were stimulated without or with insulin. Treatment with palmitate or LPS, but not Pam3CSK4 inhibited insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate -1 (IRS-1) (Fig. 4A). Moreover, insulin-stimulated phosphorylation of Akt was reduced by treatment with palmitate or LPS, but not by Pam3CSK4. This suggests that stimulation of TLR4 impairs insulin signaling pathways. To confirm the inhibitory action of TLR4 on insulin signaling in primary endothelial cells, HAECs were transfected with siRNA for scrambled or TLR4. Then insulin stimulated phosphorylation of eNOS and Akt was examined. Treatment with palmitate inhibited insulin stimulated phosphorylation of eNOS and Akt, which was restored by knock-down of TLR4 (Fig.
To examine the role of TLR4 in impairment of insulin-stimulated NO production, bovine aortic endothelial cells (BAEC) were transfected with vector only or dominant negative DNA construct of TLR4 (TLR4-DN), and then examined for the insulin-stimulated NO production by using fluorescent NO specific dye, DAF2-DA as we previously reported (22, 26). Insulin stimulated NO production was inhibited by treatment with palmitate. Overexpression of TLR4-DN partially restored the insulin stimulated NO production in the presence of palmitate (Fig. 4E).

**TLR4 knockout mice are protected from HFD-induced endothelial dysfunction** – Vascular action of insulin is associated with glucose disposal in skeletal muscle, which suggests the coordinating relationship between insulin action and endothelial function (3, 8, 27, 30, 61). Next, we examined the vasodilatory responsiveness of vessels isolated from WT (C57BL/6J) or TLR4KO mice. We fed mice normal chow diet (NCD) or high fat diet (HFD) for 10 weeks. Mesentery arterioles were isolated from the mice and examined for the vasodilatory responses to various stimuli, including insulin, acetylcholine (Ach), or sodium nitroprusside (SNP). Dilation of the vessels isolated from WT-HFD mice was impaired in response to insulin compared to those isolated from WT-NCD mice. In contrast, the vessels isolated from TLR4KO-HFD mice similarly dilated in response to insulin as those isolated from TLR4KO-NCD or WT-NCD mice (Fig. 5A). Interestingly, there was no difference in vasodilatory responses to Ach (Fig. 5B) or SNP (Fig. 5C) between groups. These results suggest that TLR4 mediates the HFD-induced impairment of vasodilator actions of insulin specifically.

**Impairment of vasodilator action of insulin by palmitate is reversed by treatment with TUDCA** – TUDCA is a well-known chemical chaperon which ameliorates ER stress (45). Because SFA-induced ER stress is mediated by TLR4, we examined whether suppressed ER stress by TUDCA improves vasodilator action of insulin or not. Infusion of palmitate impaired the vasodilator
action of insulin, and pre-treatment with TUDCA prevented the impairment of vasodilation (Fig. 6A). By contrast, neither palmitate nor TUDCA affected Ach- or SNP-stimulated vasodilation. Thus, palmitate-induced ER stress may affect insulin signaling pathways in vascular endothelial cells.

**DISCUSSION**

In the present study, we demonstrate that SFA impairs vasodilator actions of insulin through a TLR4-induced ER stress. This is the first report suggesting the link between TLR4-induced ER stress and the insulin-stimulated vasomotor activities. Moreover, TUDCA may be a potential treatment not only for fatty liver disease but also for the diabetic vascular complications in which ER stress plays important roles in pathogenesis of these cardiometabolic disorders.

*SFA stimulates pro-inflammatory responses through a TLR4-dependent mechanism* – SFAs including palmitate stimulate TLRs, which contributes to endothelial dysfunction and insulin resistance (22, 24, 34, 53, 60). Both TLR2 and TLR4 are known to be stimulated by SFAs (33, 34). One study suggests that the activation of TLRs by SFA could be due to contamination of endotoxins in bovine serum albumin (13). However, the level of endotoxin in the reagents used in this study was negligible (described in the Materials and Methods), and a more recent study strongly confirms that activation of TLR2 and TLR4 are fatty acids specific, because SFA still activates TLRs in the presence of polymixin B, a lipopolysaccharide sequester (21). We (Fig. 1 & 2) and others reported that deficiency of TLR2 or TLR4 prevents SFA-induced pro-inflammatory response in vascular endothelial cells (5, 22, 24). Interestingly, there is no additive effect when either TLR2 or TLR4 was deleted. This suggests that SFA-stimulated pro-inflammatory responses require both TLR2 and TLR4.
Receptor dimerization is the most proximal step in TLR activation, and palmitate induces dimerization of TLR2 with TLR1 and recruitment of TLR2/1 into lipid rafts which serve as a platform for the interaction of the receptor with the other signaling molecules to activate the downstream signaling pathways (54). Thus, recruitment of TLRs to lipid raft is a crucial mechanism for pro-inflammatory responses, and treatment with polyunsaturated fatty acids or apoprotein A-I attenuated the recruitment of TLRs to lipid raft (4, 64). These suggest that SFA may contribute to the microenvironment of plasma membrane promoting the activation of TLRs. Further studies warrant the detailed mechanisms by which SFA specifically activates TLRs.

**SFA stimulates ER stress through a TLR4-dependent mechanism** – There is a close association between ER stress and inflammation, which contributes to obesity-induced metabolic disorders as well as atherosclerosis (20, 31, 51, 59, 68). Imbalanced ER homeostasis causes apoptosis and activation of macrophages. Deficiency of TLR2 or TLR4 attenuated ER-stress-induced cell death and atherosclerosis in LDLR KO mice (51). Failure of adaptive response to ER stress leads to unfolding protein response (UPR), which activates JNK and NF-κB (46, 68). However, it has been unknown whether similar mechanisms exist in vascular endothelial cells. We observed that SFA stimulates ER stress through a TLR4-dependent mechanism (Fig. 2), which is consistent with the previous reports in macrophages, hepatic tissues, and hypothalamic neurons (36, 46, 51, 68). Knock-down of TLR4 reduced not only ER stress but also pro-inflammatory responses. These suggest that activation of TLR4 may perturb cellular homeostasis leading to ER stress and pro-inflammatory responses, and these two stress responses are closely associated. One potential mechanism is the activation of double-stranded RNA-dependent protein kinase (PKR), which is a nutrient sensor that transmits signaling to energy metabolism and inflammation (43). Further investigations are needed to clarify whether PKR contributes to SFA-induced inflammation and
endothelial dysfunction. Future studies may elucidate the mechanistic link between ER stress and inflammation.

**TLR4 knockout mice are protected from HFD-induced vasodilator actions of insulin** – TLR4 knockout mice is protected from atherosclerosis and inflammatory responses in aorta and macrophages (10, 39, 51). Moreover, TLR4 is involved in impairment of insulin signaling in endothelial cells through activation of NADPH oxidase (24, 35). In the present study, we have demonstrated that lack of TLR4 protects impairment of vasodilator actions of insulin (Fig. 4 & 5). Insulin-stimulated vasomotor activity is important for capillary recruitment in skeletal muscle, which contributes to glucose uptake and insulin sensitivity (3, 42, 48, 67). Since insulin stimulated capillary recruitment in skeletal muscle facilitates delivery of nutrients and hormones, NO-mediated vascular function plays an important role in energy metabolism (30). Thus, TLR4-mediated impairment of endothelial function may contribute to the HFD-induced insulin resistance, which is consistent with the study demonstrating that TLR4 knockout mice are protected from HFD-induced insulin resistance (53). Thus, our results demonstrate the potential role of TLR4 linking vascular inflammation and impaired glucose homeostasis. Taken together, the results presented here support the concept that SFA-induced impairment of vascular actions of insulin is one of the mechanisms for the obesity-induced insulin resistance (30).

We observed that HFD caused impairment of insulin-stimulated vasorelaxation (Fig. 5A), which was abolished by deletion of TLR4. Interestingly, HFD affected neither Ach- nor SNP-stimulated vasorelaxation (Fig. 5B &C). This result suggests that the impaired vasodilator actions of insulin may precede endothelial dysfunction (determined by the response to Ach) and vascular wall damage. The duration of HFD may not be severe enough to exhibit the impaired response to Ach, but may be functionally impaired in responsiveness to insulin. This may be
Clinically important to diagnose insulin resistance/endothelial dysfunction prior to develop serious diseases including diabetes and atherosclerosis.

**Impairment of vasodilator action of insulin by palmitate is reversed by treatment with TUDCA**

ER stress causes accumulation of misfolded proteins due to insufficient chaperones, which triggers cellular stress. TUDCA is a chemical chaperone that can reduce ER stress (46). Treatment with chemical chaperone may promote proper folding of proteins which leads to reduction of ER stress and inflammatory responses. TUDCA can restore the insulin sensitivity and promote glucose uptake in both human subjects and ob/ob mice (23, 45, 46). We observed that pre-treatment with TUDCA restored vasodilator action of insulin (Fig. 6A). It is possible that the restored vascular action of insulin may contribute to enhanced glucose uptake and insulin sensitivity. The specific molecular target of TUDCA and the detailed mechanisms by which ER stress affects insulin signaling are unknown. Future studies warrant the link between ER stress and insulin signaling pathways that affects both cardiovascular and metabolic functions.

In summary, we have demonstrated that SFA induces ER stress through a TLR4-mediated mechanism in vascular endothelium, and that reduction of ER stress restores vasodilator actions of insulin. These results support a notion that TLR4 and ER stress are important therapeutic targets to treat/prevent metabolic and cardiovascular disorders.
Table 1

### Primers for RT-PCR

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### DsiRNA

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**FIGURE LEGENDS**

**Figure 1. Palmitate stimulates pro-inflammatory responses through a TLR4-dependent mechanism.** HAECs were transfected with siRNA for TLR4 or scrambled, and then incubated for 48 hrs. Cells were treated with palmitate (FA, 200 µM, 6 hrs), and total RNA was isolated. The RNA was subjected to RT-PCR analysis with the specific primers indicated. Treatment with palmitate stimulated pro-inflammatory responses and knock-down of TLR4 blunted the palmitate-stimulated upregulation of pro-inflammatory gene expressions. The quantification of 3 independent experiments is shown in bar graphs (mean ± SEM). *** p <0.001, ** p <0.01, and * p <0.05 indicate that the samples are statistically different.

**Figure 2. Palmitate stimulates pro-inflammatory signaling molecules through a TLR4-dependent mechanism.** HAECs were transfected with siRNA for TLR4 or scrambled, and then incubated for 48 hrs. Cells were treated with palmitate (FA, 200 µM, 30 min), and cell lysate was isolated. The cell lysate was subjected to a western blotting with the specific antibodies. Treatment with palmitate stimulated phosphoprylation of JNK and IкBα, which was blunted by knock-down of TLR4. The quantification of 3 independent experiments is shown in bar graphs (mean ± SEM). *** p <0.001, ** p <0.01, and * p <0.05 indicate that the samples are statistically different.

**Figure 3. Palmitate stimulates ER stress through a TLR4-dependent mechanism.** HAECs were transfected with siRNA for TLR4 or scrambled, and then incubated for 48 hrs. Cells were treated with palmitate (FA, 200 µM, 6 hrs), and total RNA (A) or cell lysate (B) was isolated. The RNA was subjected to RT-PCR, and the cell lysate was subjected to a western blotting. Treatment with palmitate stimulated splicing of XBP-1 and phosphorylation of eIF-2α. Knock-down of TLR4 blunted the palmitate-stimulated XBP-1 splicing and the phosphorylation of
eIF2α. The quantification of 3 independent experiments is shown in bar graphs (mean ± SEM). *** p <0.001 and ** p <0.01 indicate that the samples are statistically different.

**Figure 4. Activation of TLR4 impairs insulin signaling.** A-C. HEK293 cells were stably transfected with TLR4 and MD2 as described in the Material and Methods. The cells were serum starved with media containing 0.1% horse serum for 2 hrs and then treated with palmitate (FA, 100 μM), Pam3Csk4 (Pam3, 1 μg/ml), or LPS (1 μg/ml) for 24 hours, followed by treatment with insulin (100 nM, 5 min). Cell lysates were collected and subjected to a western blot analysis. Blots were incubated with the indicated antibodies. Representative blots from three independent experiments. Tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) was detected by blotting a whole lysate with anti-phosphotyrosine antibody. Phosphorylation of Akt was detected by using anti-pAkt (A). Tyrosine phosphorylation was normalized to β-actin (B). Phospho-Akt is normalized to total Akt (C). The quantification of 3 independent experiments is shown in bar graphs (mean ± SEM). *** p <0.001, ** p <0.01, and * p <0.05 indicate that the samples are statistically different compared to insulin treated samples. D. BAECs were transfected with scrambled or siRNA for TLR4. Two days later, cells were treated with either BSA or palmitate (200 μM, 4 hrs). Cell lysates were collected and subjected to an immunoblotting. Representative blots from three independent experiments. The demarcated lines are due to the noncontiguous lanes, but they are from a single gel. E. BAECs were transiently transfected with vector (pcDNA 3.1) or dominant negative construct of TLR4 as described in Methods. After cells were incubated for 48 hrs, cells were serum starved for 2 hrs, and then cells were loaded with the NO-specific fluorescent dye DAF2-DA. Cells were pre-treated with BSA or palmitate (200 μM, 4 hrs) and then treated without or with insulin (100 nM) for 20 min. After insulin treatment, cells were fixed with 2% paraformaldehyde and visualized with an
epifluorescent microscope as described in Methods. Emission of green fluorescence is indicative of NO production. Experiments shown are representative of those that were independently repeated at least 3 times.

Figure 5. TLR4 knockout mice are protected from HFD-induced endothelial dysfunction. C57BL/6 mice or TLR4KO mice were fed normal chow diet (10% calorie from fat) or high fat diet (60% calorie from fat) for 10 weeks. Mesentery arterioles were isolated and subjected to videomicroscopic analysis for vasodilation in response to various stimulators, including insulin (A), acetylcholine (Ach) (B), and sodium nitroprusside (SNP) (C). Vessels isolated from WT mice (C57BL/6J) HFD were impaired to dilate in response to insulin compared to those isolated from WT NCD mice. By contrast, arterioles isolated from both NCD and HFD TLR4 knockout mice similarly dilated in response to insulin compared to those isolated from WT NCD mice. However, there were no differences in responses to Ach or SNP. The data suggests that HFD specifically impairs insulin-stimulated vasodilation through a TLR4-dependent mechanism. ### p <0.001, # p < 0.05 (WT+NCD vs. WT+HFD), *** p <0.001 (WT+HFD vs. TLR4KO+HFD)

Figure 6. Impairment of vasodilatory action of insulin by palmitate is reversed by treatment with TUDCA (ER stress inhibitor). Mesentery arterioles were isolated from 6 week old mice. Vascular activities were measured by treatment with various stimulators, including insulin (A), acetylcholine (Ach) (B), and sodium nitroprusside (SNP) (C). Intraluminal infusion of palmitate (200 μM) impaired vasodilator action of insulin compare to BSA (control) infusion. Pre-treatment with TUDCA (500 μM, 30 min before palmitate infusion) prevented the effect of palmitate. By contrast, there were no differences between groups when the vessels were treated with Ach or SNP. The results suggest that palmitate-induced ER stress may be the mechanism to cause impairment of vasodilation in response to insulin, and the attenuation of ER stress by
TUDCA restores the vasodilator action of insulin. ### p <0.001, ## p < 0.01 (TUDCA+FA vs. FA), *** p <0.001 (FA vs. BSA)

**Acknowledgements**

This study was supported by the American Diabetes Association (1-09-JF-33; 1-12-BS-99 to J.K; 1-13-BS-150 to M.J.Q), American Heart Association (13GRNT17220057 to J.K), and UAB diabetes research center sponsored pilot and feasibility program supported by National Institutes of Health (P60 DK-079626), and UAB Comprehensive Diabetes Center, and USDA-ARS program project (5306-51530-017-00D) and USDA/NIFA competitive grant (2013-03477 to D.H.H.).

**Disclosures**

None

**Author contributions**

J.K. designed experiments, analyzed the data and wrote and edited the manuscript. H.J. performed experiments and analyzed data. D.H. helped interpretation of data and edited the manuscript.
REFERENCES


Figure 4

A.

Insulin (100 nM, 5 min)

- BSA  - FA  - Pam3  - LPS

pY (IR)

pY (IRS-1)

IRS-1

p-AKT S473

AKT

β-actin

B.

pY

Increase Fold

BSA  BSA+Ins  FA  FA+Ins  Pam3  Pam3+Ins  LPS  LPS+Ins

***  **

C.

pAkt

Increase Fold

BSA  BSA+Ins  FA  FA+Ins  Pam3  Pam3+Ins  LPS  LPS+Ins

*  ***
Figure 5

A. % Dilation vs. Insulin [log M]

B. % Dilation vs. Ach [log M]

C. % Dilation vs. SNP [log M]
Figure 6

A. % Dilation vs. Insulin [log M]

B. % Dilation vs. Ach [log M]

C. % Dilation vs. SNP [log M]