Toll-Like Receptor 4 Mutation Protects Obese Mice Against Endothelial Dysfunction by Decreasing NADPH Oxidase Isoforms 1 and 4

Chao-Fan Liang, Jacky TC Liu, Yu Wang, Aimin Xu, Paul M. Vanhoutte

Objective—To analyze the role of toll-like receptor 4 in modulating metabolism and endothelial function.

Approach and Results—Type 2 diabetic mice with mutated toll-like receptor 4 (DWM) were protected from hyperglycemia and hypertension, despite an increased body weight. Isometric tension was measured in arterial rings with endothelium. Relaxations to acetylcholine were blunted in aortae and mesenteric arteries of Lepr<sup>db/db</sup> mice, but not in DWM mice; the endothelial NO synthase dimer/monomer ratio and endothelial NO synthase phosphorylation levels were higher in DWM preparations. These differences were abolished by apocynin. Contractions to acetylcholine (in the presence of L-NAME) were larger in carotid arteries from Lepr<sup>db/db</sup> mice than from DWM mice and were inhibited by indomethacin and SC560, demonstrating involvement of cyclooxygenase-1. The release of 6-ketoprostaglandin F<sub>1α</sub> was lower in DWM mice arteries, implying lower cyclooxygenase-1 activity. Apocynin, manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin, catalase, and diethyldithiocarbamate inhibited endothelium-dependent contractions. The mRNA and protein levels of NADPH oxidase isoforms NOX1 and NOX4 were downregulated in DWM mice arteries. The in vivo and in vitro administration of lipopolysaccharide caused endothelial dysfunction in the arteries of wild-type, but not toll-like receptor 4–mutated mice.

Conclusions—Toll-like receptor 4 plays a key role in obesity and diabetes-associated endothelial dysfunction by increasing oxidative stress. (Arterioscler Thromb Vasc Biol. 2013;33:777-784.)

Key Words: endothelium-derived contracting factor • nitric oxide • obesity • proinflammatory cytokines • systolic arterial blood pressure

Obesity and diabetes mellitus are associated with augmented circulating levels of free fatty acids, low-grade chronic inflammation, and endothelial dysfunction attributable to impaired release of endothelium-derived relaxing and augmented production of endothelium-derived contracting (EDCF) factors. Thus, the bioavailability of NO is reduced by obesity and diabetes mellitus, and relaxations attributable to endothelium-derived hyperpolarization (EDH) are impaired at the early stage of diabetes mellitus. Finally, increased release of endothelium-derived vasoconstrictor prostanoids and reactive oxygen species (ROS) also contributes to endothelial dysfunction in obesity and diabetes mellitus.

Proinflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin 6 [IL-6], resistin, and lipocalin-2) link obesity to metabolic and vascular dysfunction. Bacterial endotoxin lipopolysaccharide (LPS) and saturated fatty acids share as pattern recognition target toll-like receptors 4 (TLR4), which activate inflammatory pathways, induce cytokine expression in the endothelium, and augment the expression of NADPH oxidase in macrophages. The absence of TLR4 protects mice against obesity-induced insulin resistance. NADPH oxidase is a major source of endothelial ROS. ROS decrease the bioavailability of NO and facilitate the production of EDCF.

It is unknown whether or not TLR4 signaling contributes to the impaired NO- and EDHF-mediated relaxations and the amplified EDCF-mediated contractions seen in obesity and diabetes mellitus. Therefore, the present experiments tested the hypothesis that TLR4 signaling mediates, at least in part, the deleterious effects of diet-induced and genetic obesity leading to endothelial dysfunction.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

General Characteristics
There were no significant differences in food intake between wild-type (WT) and TLR4<sup>−/−</sup> mice (Figure 1A in the online-only Data Supplement). The high-fat diet evoked comparable increases in body weight in 12-week-old WT and TLR4<sup>−/−</sup> mice.
In DWM mice, the food intake and the body weight were higher starting at the age of 8 weeks (Figure IC in the online-only Data Supplement) compared with Leprdb/db mice. Both feeding (Figure IC in the online-only Data Supplement) and fasting (Figure ID in the online-only Data Supplement) glucose levels of DWM mice were significantly lower than those of Leprdb/db mice. After injection of glucose, the glycemia rose more slowly and returned back to normal more quickly in DWM mice than in Leprdb/db mice (Figure IE and IF in the online-only Data Supplement). After injection of insulin, blood glucose dropped more quickly in DWM mice than in Leprdb/db mice (Figure IG and IH in the online-only Data Supplement).

The mRNA expressions of IL1-β, IL-6, and TNF-α were significantly lower in the epididymal fat of DWM mice compared with Leprdb/db mice (Figure II in the online-only Data Supplement).

Systolic arterial blood pressures were comparable between 16-week-old lean WT and TLR4−/− mice. WT mice were fed high-fat diet, and Lepr db/db mice fed standard chow exhibited an elevated systolic arterial blood pressure (Figure IIIA in the online-only Data Supplement). DWM mice showed significantly lower systolic arterial blood pressures than Leprdb/db mice (Figure IIIA in the online-only Data Supplement).

**Vascular Responsiveness**

**Mutation of TLR4**

Relaxations to increasing concentrations of acetylcholine (10−10 to 10−4 mol/L) were reduced significantly in aortae of 16-week-old WT mice compared with TLR4−/− preparations (Figure 1).

Increasing concentrations of acetylcholine (in the presence of L-NAME [10−4 mol/L; nonselective NOS inhibitor] and indomethacin [10−5 mol/L; nonselective cyclooxygenase inhibitor] to assess responses attributable to EDH) caused relaxations in mesenteric arteries, which were significantly larger in preparations of TLR4−/− than in those of control mice (Figure 1).

In the presence of L-NAME (10−4 mol/L, to optimize endothelium-dependent contractions), acetylcholine induced increases in tension in the quiescent carotid arteries of WT and TLR4−/− mice (Figure 1). These contractions were significantly attenuated in TLR4−/− mice arteries.

**High-Fat Diet**

After 12 weeks of high-fat diet, relaxations to acetylcholine (10−10 to 10−4 mol/L) were significantly impaired in aortae or mesenteric arteries (in the presence of L-NAME and indomethacin) of WT mice (Figure 1). The high-fat diet did not significantly affect relaxations to acetylcholine in either aortae or mesenteric arteries of TLR4−/− mice (Figure 1).

Contractions induced by acetylcholine in the presence of L-NAME were enhanced significantly in WT carotid arteries after high-fat feeding (Figure 1).

**Double Mutation of Leptin Receptor and TLR4**

**Vascular Smooth Muscle**

Contractions to 60 mmol/L KCl were not significantly different in the aortae of 12-week-old Leprdb/db and DWM mice (1.83±0.49 and 1.79±0.36 g, respectively). Likewise, contractions to increasing concentration of U46619 were not significantly different in Leprdb/db and DWM mice aortae without endothelium (Figure IIIC in the online-only Data Supplement).

Relaxations to increasing concentrations of sodium nitroprusside were not significantly different between Leprdb/db and DWM mice aortae (Figure IIIB in the online-only Data Supplement).

**NO-Dependent Relaxations and eNOS Coupling**

The relaxations to acetylcholine (10−10 to 10−4 mol/L) were significantly larger in DWM compared with Lepr db/db mice aortae (Figure 2A). After incubation with apocynin (10−4 mol/L; antioxidant), the relaxations to acetylcholine were potentiated only in Leprdb/db mice aortae, and the difference in relaxations...
between the 2 strains was no longer significant (Figure 2A). The relaxations to acetylcholine were abolished by L-NAME (10^{-4} \, \text{mol/L}) in the aortae of both strains (Figure IIIE in the online-only Data Supplement).

The basal and the acetylcholine-evoked phosphorylation of endothelial NO synthase (eNOS) at Serine 1177 was significantly higher in DWM than in Leprdb/db mice aortae (Figure 2B). Apocynin enhanced the acetylcholine-evoked phosphorylation of eNOS only in Leprdb/db preparations. The expression of total eNOS was comparable in Leprdb/db and DWM preparations and not affected by apocynin (Figure 2B).

The eNOS dimer/monomer ratio was significantly higher in DWM than in Leprdb/db mice aortae (Figure 2C). Apocynin increased the eNOS dimer levels and decreased those of eNOS monomer in Leprdb/db aortae to comparable levels as those observed in DWM preparations (Figure 2D). The eNOS dimer and monomer levels were not altered significantly by apocynin in DWM preparations (Figure 2D).

**Endothelium-Dependent Contractions**

In the presence of L-NAME, acetylcholine evoked concentration-dependent contractions in quiescent carotid arteries of 12-week-old Leprdb/db mice (Figure 3A). The increases in tension caused by acetylcholine were significantly smaller in DWM preparations (Figure 3A). The contractions were abolished by apocynin (Figure 3A and 3C). Removal of the endothelium, as well as incubation with indomethacin, SC560 (3\times10^{-7} \, \text{mol/L}; selective cyclooxygenase [COX]-1 inhibitor) and S18886 (10^{-7} \, \text{mol/L}; thromboxane-prostanoids receptor antagonist), but not NS398 (10^{-6} \, \text{mol/L}; preferential COX-2 inhibitor), significantly inhibited the acetylcholine-induced contractions (Figure V in the online-only Data Supplement). Diphenyliodium (10^{-5} \, \text{mol/L}; flavoenzyme oxidoreductases inhibitor), diethyldithiocarbamate (10^{-4} \, \text{mol/L}; superoxide dismutase inhibitor), catalase-polyethylene glycol (1200 U mL^{-1}), and manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (10^{-4} \, \text{mol/L}; cell permeable superoxide dismutase mimetic) significantly decreased the endothelium-dependent contractions to acetylcholine in both type of preparations, whereas superoxide dismutase (120 U mL^{-1}) and deferoxamine
LPS Administration

The glucose and insulin tolerances were comparable in WT mice 4 hours after the in vivo intraperitoneal injection of either vehicle or LPS (25 mg/kg; Figure VI in the online-only Data Supplement).

NO-Dependent Relaxations

The acetylcholine-induced concentration-dependent relaxations were not significantly different between the aortae of 8-week-old WT and TLR4<sup>−/−</sup> mice (Figure 5). The in vivo injection of LPS significantly attenuated the relaxations to acetylcholine in WT, but not in TLR4<sup>−/−</sup> preparations (Figure 5). The acetylcholine-induced relaxations in WT aortae were normalized by apocynin (10<sup>−4</sup> mol/L), which did not significantly affect the response in TLR4<sup>−/−</sup> preparations (Figure 5). The injection of LPS did not significantly affect the relaxations to sodium nitroprusside (Figure VII in the online-only Data Supplement).

In vitro incubation with LPS (10<sup>−6</sup> mol/L for 4 hours) significantly attenuated the relaxations to acetylcholine in WT, but not in TLR4<sup>−/−</sup> arteries (Figure 6).

EDH-Mediated Relaxations

In mesenteric arteries (in the presence of L-NAME and indomethacin) of 8-week-old WT and TLR4<sup>−/−</sup> mice, acetylcholine induced comparable concentration-dependent and endothelium-dependent relaxations (Figure 5). The in vivo injection of LPS reduced the relaxations to acetylcholine in WT, but not in TLR4<sup>−/−</sup> arteries (Figure 5). The in vitro administration of apocynin prevented this inhibitory effect of LPS (Figure 5).

In vitro incubation with LPS significantly reduced the relaxations to acetylcholine in WT, but not in TLR4<sup>−/−</sup> arteries (Figure 6).

Endothelium-Dependent Contractions

In the presence of L-NAME (10<sup>−4</sup> mol/L), acetylcholine evoked comparable and minimal contractions in rings with endothelium of carotid arteries of lean WT mice and TLR4<sup>−/−</sup> mice (Figure 5). After the in vivo injection of LPS, it induced significantly larger increases in tension in WT, but not in TLR4<sup>−/−</sup> preparations, which were inhibited by apocynin (Figure 5). However, in vivo injection of LPS did not significantly affect the contractions to the thromboxane-prostanoids receptor agonist U46619 (Figure VIII in the Supplement).

After in vitro incubation with LPS, acetylcholine induced significantly larger increases in tension in WT preparations only (Figure 6).

Discussion

The current experiments were undertaken to determine whether or not TLR4, a major target for the initiation of inflammatory responses,<sup>13</sup> contributes to the endothelial dysfunction resulting from diet-induced obesity and diabetes mellitus. Responses of arteries from WT mice were compared with those obtained in preparations from mice with mutated, nonfunctional TLR4, subjected to a high-fat diet. Furthermore, a unique animal model was created, with combined nonfunctional mutation of the leptin and TLR4 receptors. Although these DWM mice displayed a significantly heavier body weight, they were protected from hypertension and
hyperglycemia demonstrating that overweight, per se, does not cause these pathological changes. They can be attributed to the decreased production of cytokines illustrated by the lower expression of TNF-α, IL-1β, and IL-6 in the DWM mice adipose tissue. The lower plasma level of free fatty acids and cholesterol, also attributable to the TLR4 mutation,14 explained why these animals did not develop the equivalent of a metabolic syndrome. The enhanced insulin sensitivity in DWM mice directly illustrates the link between TLR4 and insulin resistance.

The present study confirms that high-fat diet and diabetes mellitus increase arterial blood pressure in mice and demonstrates that loss of function of TLR4 prevents obesity- and diabetes-induced hypertension but does not affect this parameter in animals receiving standard diet.

Three types of endothelium-dependent responses were investigated in the present study.

First, NO-mediated acetylcholine-induced relaxations were determined in aortae in which, endothelium-dependent relaxations are attributable exclusively to the production of NO by eNOS.17 The involvement of NO was confirmed by the abolition of the responses by L-NAME, a nonselective inhibitor of NO synthases. In the aortae of 16-week-old WT mice given high-fat diet, or those of Leprdb/db mice, relaxations to acetylcholine, but not those to sodium nitroprusside, were blunted, demonstrating endothelial dysfunction. However, such relaxations were not altered in preparations of TLR4−/− or DWM mice, suggesting that TLR4 loss-of-function protects against the reduction in NO bioavailability attributable to diet-induced obesity and diabetes mellitus. The latter reduction is attributable to TLR4-induced impairment of eNOS activation. This conclusion is supported by the Western blotting data in Leprdb/db mice demonstrating a reduced phosphorylation both under basal conditions and on stimulation with acetylcholine, and an increased eNOS monomer level, 2 measures of the activation of the enzyme.17 In mice, uncoupling of eNOS can be caused by hypercholesterolemia.18 The present study demonstrates that loss of function of TLR4 rescues diabetic obese mice from the impaired relaxations. The likely mechanism underlying this effect is a reduced superoxide anion production leading to prevention of eNOS uncoupling.19–21 This interpretation is supported by the observation that in Lepr db/db aortae the antioxidant apocynin, which scavenges superoxide anions,10 recoupled eNOS allowing phosphorylation of the enzyme on exposure to acetylcholine, thus potentiating relaxations to the muscarinic agonist.

Second, the involvement of endothelium-dependent hyperpolarization was assessed by determining the inhibitory effect of incubation with 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole and 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-Trietheno-11,7-Metheno-7H-Dibenzo[b,m]5,13-Diium Ditrifluoroacetate hydrate on relaxations to acetylcholine, obtained in the presence of L-NAME and indomethacin.22 The present findings confirm that EDH-mediated relaxations are impaired in arteries of obese and diabetic mice.2 Activation of TLR4 triggers the production of inflammatory cytokines.7 In arteries of diabetic mice, TNF-α deficiency reverses the blunting of EDH-mediated relaxations.4 Taken in conjunction with the reduced level of TNF-α, IL-1β, and IL-6 observed in the adipose tissue of DWM mice, the prevention of the impairment of EDH-mediated relaxations observed in their arteries illustrates that
TLR4 signaling contributes to this aspect of endothelial dysfunction resulting from obesity and diabetes mellitus.

An elevated glucose level inhibits acetylcholine-induced, EDH-mediated responses in the rat mesenteric artery because of the increased production of ROS. The administration of the antioxidant apocynin prevented the reduction in EDH-mediated relaxation, suggesting that the impaired response in arteries with functional TLR4 signaling also results from an increased ROS production.

Third, the aberrant production of vasoactive factors causing endothelial dysfunction includes the increased release of EDCFs from endothelial cells. In the mouse, EDCF-mediated responses are most pronounced in the carotid artery, and therefore this preparation was selected to examine endothelium-dependent...
contractions. Various arachidonic acid metabolites, including thromboxane A2, prostaglandin F2α, and prostacyclin, contribute to endothelium-dependent contractions that are ultimately attributable to activation of thromboxane-prostanoids receptors of the vascular smooth muscle.26 In the present study, the levels of 6-ketoPGF1α, the stable metabolite of prostacyclin, were measured to estimate the production of EDCF.24,26,27

The findings that the endothelium-dependent contractions to acetylcholine were abolished by indomethacin and a thromboxane-prostanoids receptor antagonist (S18886) demonstrate an EDCF-mediated response.24,26,27 Augmented endothelium-dependent contractions were observed in carotid arteries of older or high-fat fed WT and Leprdb/db mice, but not in TLR4−/− and DWM preparations, implying an amplification of the phenomenon by functional TLR4. The acetylcholine-induced endothelium-dependent contractions were blocked by a selective COX-1, but not by a COX-2 inhibitor, confirming the key role of COX-1 in EDCF-mediated responses in mice.29 TLR4 mutation did not significantly affect the COX-1 expression at either the mRNA or protein levels, implying that it does not modulate the presence of the enzyme. However, because the production of 6-ketoPGF1α was increased in Leprdb/db arteries, TLR4 must upregulate COX-1 activity. The antioxidant apocynin reduced the production of 6-ketoPGF1α, and hence the endothelium-dependent contractions,24,26,27 suggesting that ROS play a key role in activating cyclooxygenase to generate prostanoids when TLR4 is functional. Thus, the reduced endothelium-dependent contractions in the carotid arteries of the DWM mice are consistent with the lower production of ROS suggested by the lucigenin-chemiluminescence measurements in isolated membrane preparations of these blood vessels.28,29 In small mesenteric arteries of diabetic mice, the enhanced contractile activity is also associated with increased oxidative stress and cyclooxygenase production.28 The lower ROS production in DWM mice arteries is accompanied by a lesser mRNA expression and protein presence of NOX1 and NOX4, the major isoforms of NADPH oxidase that contribute to oxidative stress in the vascular wall. LPS, the prototypical agonist of TLR4, stimulates the production of superoxide anions in cultured endothelial cells, and this response is reduced by silencing RNAs for either NOX1 or NOX4 (Figure IX in the online-only Data Supplement). These observations indirectly support the interpretation that the decreased ROS production in DWM mice arteries may well result from a lesser activity of NADPH oxidase.

The finding that the endothelium-dependent contractions were prevented by the antioxidant apocynin and the inhibitor of flavoenzyme oxidoreductases (including NADPH oxidase) diphenylidium is consistent with the key initial role of ROS in the EDCF-mediated contraction. The inhibitory effects of the cell permeable agents, diethyldithiocarbamate, catalase-polyethylene glycol, and manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin, observed in the present experiments imply that the dismutation of superoxide anions to H2O2 is also required. The observation that the addition of exogenous superoxide dismutase, which does not permeate cell membranes, did not prevent the endothelium-dependent contractions to acetylcholine, in confirmation of earlier studies in the rat aorta, indicates that the dismutation occurs intra-cellularly. The comparable contractions to exogenous H2O2 observed in the carotid arteries of Leprdb/db and DWM mice suggest that augmented levels of free radicals in the endothelial cells rather than their direct effect on vascular smooth muscle play a crucial role in the dysfunction observed in Leprdb/db preparations.

The present study thus demonstrates that loss-of-function in TLR4 protects mice from endothelial dysfunction resulting from obesity, whether genetic or diet induced, by both potentiating endothelium-dependent relaxations and decreasing endothelium-dependent contractions. These protective effects of the TLR4 mutation can be attributed to a reduced production of ROS because the different aspects of endothelial dysfunction observed in obese/diabetic mice with functional TLR4 are prevented by apocynin. Elevated ROS formation in the vascular wall is a key feature of cardiovascular disease and a likely contributor to endothelial dysfunction and vascular inflammation.33 ROS generated by NOX are the initial source of endothelial dysfunction in diabetes mellitus.5,9,10 NOX1 contributes significantly to gastrointestinal inflammation, hypertension, and restenosis after angioplasty.33

LPS is the prototypical ligand for TLR4 and triggers the signaling cascade leading to the activation of nuclear factor-κB and the production of proinflammatory cytokines, including TNF-α and IL-1β.8 Indeed, mutation of the TLR4 gene prevents LPS signal transduction in vitro and in vivo.34 Chronic elevation of the circulating levels of LPS exists in obese subjects and contributes to insulin resistance and its related cardiometabolic complications.35,36 The present experiments demonstrate that LPS administration in vivo to lean WT mice does not change glucose tolerance and insulin sensitivity but impairs endothelium-dependent relaxations and enhances endothelium-dependent contractions, mimicking the vascular phenotype observed in obese/diabetic mice. However, LPS fails to elicit these vascular responses in mice with mutation of TLR4, implying that the derogative effects of LPS on vascular tone are TLR4 dependent. These findings suggest that TLR4 activation-induced endothelial dysfunction is a direct effect and not secondary to metabolic changes. This conclusion is strengthened by the demonstration that in vitro administration of LPS had identical effects on endothelial function as its in vivo injection, and this again only in WT arteries. The ex vivo reversal by apocynin of these responses induced by LPS indicates again that ROS are the key link between TLR4 and endothelial dysfunction.

In conclusion, the present findings suggest that activation of TLR4 promotes the transcription of NADPH oxidase 1 and 4, resulting in elevated reactive oxidative stress. In endothelial cells, the increased level of ROS reduces eNOS coupling leading to a reduced NO-production and bioavailability, impairs EDH-mediated responses, and increases the activity of COX-1 with augmented EDCF-mediated contractions. These effects combine to initiate endothelial dysfunction in arteries of obese and diabetic mice. The present study identifies TLR4 as being responsible for the endothelial dysfunction resulting from obesity and diabetes mellitus.

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Disclosures

None.

References


Significance

This study reveals that loss-of-function of toll-like receptor 4 in mice can alleviate dietary obesity- and diabetes-associated endothelial dysfunction by decreasing the production of reactive oxygen species (generated by NADPH oxidase isoforms 1 and 4) and vasconstrictor prostaglandins (generated by cyclooxygenase-1) in endothelial cells. Because endothelial dysfunction precedes several cardiovascular complications, toll-like receptor 4 inactivation may be a therapeutic target for the prevention and treatment of vascular disease associated with obesity and diabetes mellitus.
Toll-Like Receptor 4 Mutation Protects Obese Mice Against Endothelial Dysfunction by Decreasing NADPH Oxidase Isoforms 1 and 4

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Materials and Methods

Animals.

The procedures were approved by the Committee on the Use of Live Animals of the University of Hong Kong. Leptin receptor heterozygous (Lepr${^{db/+}}$) mice on a C57BL/6J background, C3H/HeOuJ (wild type; WT) mice and toll-like receptor 4 mutation (TLR4$^{-/-}$) mice on a C3H background were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Leptin receptor$^{+/+}$/TLR4$^{-/-}$ double mutation (DWM) mice were generated by cross-breeding male C57BL/6J Lepr$^{db/+}$ mice with female TLR4$^{-/-}$ mice. The mice were further backcrossed for at least ten generations onto the C3H background (see supplementary data). The mice were housed in a room under controlled temperature (23 ± 1°C) and 12-hours light-dark cycle, with free access to water and standard chow (LabDiet 5053; Purina Mills, Richmond, IN). Dietary obesity was induced by allowing free access to a high-fat diet (D12451; Research Diet, New Brunswick, NJ, USA) from the age of four weeks onwards. The comparisons made were between wild-type and mutation littermates from heterozygous breeders. Intraperitoneal glucose tolerance (ipGTT) and insulin tolerance (ipITT) tests were performed as described. In certain experiments lipopolysaccharide (LPS, 25mg/Kg) was given intraperitoneally four hours prior to ipGTT, ipITT or sacrifice.
Methodology

Blood pressure measurements. Arterial blood pressure was measured with an automated tail-cuff BP-2000 Blood Pressure Analysis System (Visitech Systems, Apex, NC, USA). All recordings were obtained between 15:00 and 18:00 hour, after warming the animals in a 35°C chamber for ten minutes. The mice were habituated to this procedure during three days before the actual experiments. The systolic and diastolic blood pressures were recorded and averaged from at least ten consecutive readings.

Isometric force measurement. Mice were anesthetized with pentobarbital sodium (25 mg·ml⁻¹·kg⁻¹ ip). Blood was collected from the heart, and plasma was stored at -70°C after centrifugation (10000 rpm/minute). The thoracic aorta, superior mesenteric artery and carotid arteries were dissected free, cleaned of adhering connective tissues, cut into rings (approximately 2-3 mm length), and placed in cold, modified Krebs-Ringer bicarbonate solution containing (mM) 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 D-glucose (control solution). The rings were suspended in a Mulvany-Halpern wire myograph (Model 610M, Danish Myo Technology A/S, Aarhus, Denmark) for the recording of isometric contractile force (PowerLab 4SP, ADInstruments, Colorado Springs, CO, USA). The organ
chambers were filled with warmed (37°C), aerated (95% O₂ and 5% CO₂) control solution. For aortae, the endothelium was removed by perfusion of the lumen with 0.1% Triton X-100 in control solution prior to cutting the rings. In some preparations of mesenteric arteries and carotid arteries, the endothelium was removed mechanically by gently rubbing the surface of the rings with a strand of hair. Removal of endothelium was confirmed by the absence of relaxation in response to acetylcholine (agonist at muscarinic and nicotinic receptors, 10⁻⁶ M). Rings were allowed to equilibrate for one hour at the optimal resting tension (determined in preliminary experiments; data not shown) of 0.5 g, 0.3 g and 0.5 g for aortae, mesenteric and carotid arteries, respectively. The aortae and mesenteric arteries were contracted with 9,11-dideoxy-11alpha, 9alpha-epoxymethanoprostaglandin F₂α (U46619; TP receptor agonist; 1 to 3 x 10⁻⁸ M) before being exposed to increasing concentrations of acetylcholine (10⁻¹⁰ to 10⁻⁴ M), or sodium nitroprusside (nitric oxide donor, 10⁻¹⁰ to 10⁻⁴ M). Decreases in tension were expressed as percentage of the contraction to U46619. Quiescent carotid arteries were exposed to increasing concentrations of acetylcholine (10⁻⁸ to 10⁻⁶ M). Increases in tension were expressed as a percentage of a reference contraction to potassium chloride (KCl, 60 mM) obtained at the beginning of the experiment.
Free radical production. The superoxide anion levels and NADPH oxidase activity were measured with a lucigenin chemiluminescence assay, using a Tecan M200 Plate-reader (Tecan Trading AG, Mannedorf, Switzerland) to maintain the sample temperature at 37°C. All experiments were performed in quadruplicate. Membrane fractions prepared by homogenizing blood vessels in a buffer (50 mM Tris-HCl, pH 7.4) containing a Complete™ Protease Inhibitor Cocktail, followed by centrifugation of the supernatant at 100,000 g for 45 min (Rajagopalan S, Kurz S, Muenzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG Clin Invest, 1996, 97: 1916-1923); The membrane fraction was suspended in PBS, quantified by the BCA method (Bio-rad), and NADPH oxidase activity of the membrane suspension was measured by lucigenin chemiluminescence assay as described. The basal and acetylcholine (10^-6 M)-stimulated chemiluminescence [in the absence or presence of indomethacin (non-selective COX inhibitor, 5 x 10^-6 M)] were recorded every 29 seconds for 30 minutes, and the average reading calculated. The lucigenin chemiluminescence was normalized as relative light units (RLU) per second per milligram of protein.

Production of 6-ketoprostaglandin \( F_{1\alpha} \). The release of 6-ketoprostaglandin \( F_{1\alpha} \) was measured by immunoassay. After 30 minutes of incubation at 37°C in a chamber (in the presence of L-NAME) and with and without pharmacological inhibitors, carotid
arteries rings were transferred to microcentrifuge tubes that contained 200 μl control solution and 3 x 10^{-4} M acetylcholine. Three minutes later, the tissues were removed and the solutions were frozen and stored at -80 °C (Gluais et al., 2005) until immunoassay for the determination of the content of 6-ketoprostaglandin F_{1α} according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

*Gene expression.* mRNA was detected by quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA from mice epididymal fat or carotid arteries was purified with a Trizol reagent (Invitrogen), and treated with RNase-free DNase (Qiagen, Hamburg, Germany) at 37°C for 30 minutes to remove genomic DNA. For reverse transcription, one μg of the total RNA was converted to first-strand complementary DNA in 20 μl reactions using a cDNA synthesis kit (Qiagen, Hamburg, Germany). The quantitation of target genes was performed using SYBR Green PCR Master Mix (Qiagen) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used are included in Table I.

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<tr>
<th>Gene</th>
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<td>Mice NOX1</td>
<td>CTGAGAAAGCCATTGGATCA</td>
<td>GGATGGGATTTAGCCAAGAA</td>
</tr>
<tr>
<td>Mice NOX4</td>
<td>AAGCCCATTTGAGGAGTCAC</td>
<td>GCAAACCACTGGAATGATTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TTGTCTCCTGCGACTTCAAC</td>
<td>TCTTACTCCTTGAGGCCAT</td>
</tr>
</tbody>
</table>

**Protein presence.** Protein expression was determined by Western blotting. The aortae and carotid arteries from wild type and TLR4−/− mice were homogenized in lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate) containing protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitors, 1 mg/ml leupeptin, and 2 μg/ml pepstatin A). Then, 100 μg of proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, incubated with antibodies against phosphorylated (Ser1177) and total eNOS (BD Transduction Laboratories, San Jose, CA, USA), phosphorylated p85 (Tyr458)/p55 (Tyr199) and total PI3K, phosphorylated (Ser473) and total Akt, phosphorylated and total GSK3β, β-catenin, cyclooxygenase-1 and beta-actin (Cell
Signaling, Beverly, MA, USA). The immune complexes were detected with enhanced chemiluminescence reagents from GE Healthcare (Uppsala, Sweden).

Low-temperature SDS-PAGE was performed for detection of eNOS monomers and dimers. Total proteins were incubated in loading buffer without 2-mercaptoethanol at 37°C for five minutes and subsequently subjected to 6% SDS-PAGE. Gels and buffers were equilibrated at 4°C before electrophoresis, and the buffer tank was placed in an ice bath during the procedure.

**Data analysis**

All results were derived from at least four sets of repeated experiments. To simplify the presentation of the results obtained with increasing doses of acetylcholine, in certain figures, the area under each concentration-response curve was computed and statistical significances between areas under the curves of different datasets were analyzed using Student’s *t*-test or two-way ANOVA followed by the Bonferroni *post hoc* test. Western blotting data were analyzed using the one sample *t*-test comparing mean band intensity of the treatment groups against that of controls. All statistical analysis and calculations were performed using Prism version 5 (GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered to indicate statistically significant differences.
**Drugs**

Drugs used for pharmacological experiments are given in table II. The incubation time with pharmacological inhibitors was 30 minutes.

<table>
<thead>
<tr>
<th>Full name</th>
<th>Effect</th>
<th>Concentration</th>
<th>Source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>apocynin</td>
<td>non-specific inhibitor of NADPH oxidase</td>
<td>$10^{-4}$ M</td>
<td>Calbiochem (La Jolla, CA, USA)</td>
<td>5</td>
</tr>
<tr>
<td>catalase-PEG</td>
<td>hydrogen peroxide scavenger</td>
<td>1200 U ml$^{-1}$</td>
<td>Sigma-Aldrich, St. Louis, MO, USA</td>
<td>5</td>
</tr>
<tr>
<td>deferoxamine</td>
<td>iron scavenger</td>
<td>$10^{-4}$ M</td>
<td>Calbiochem (La Jolla, CA, USA)</td>
<td>5</td>
</tr>
<tr>
<td>diethylthiocarbamate (DETCA)</td>
<td>superoxide dismutase inhibitor</td>
<td>$10^{-4}$ M</td>
<td>Sigma-Aldrich</td>
<td>5</td>
</tr>
<tr>
<td>diphenyliodonium (DPI)</td>
<td>flavoenzymes oxidoreductases inhibitor</td>
<td>$10^{-6}$ M</td>
<td>Sigma-Aldrich</td>
<td>5</td>
</tr>
<tr>
<td>indomethacin</td>
<td>non-specific inhibitor of cyclooxygenases</td>
<td>$10^{-5}$ M</td>
<td>Sigma-Aldrich</td>
<td>6</td>
</tr>
<tr>
<td>Mn (III) tetrakis (1-Methyl-4-pyridyl) pyrpyrin Pentachloride (MnTMPyP)</td>
<td>catalase mimetic</td>
<td>$10^{-5}$ M</td>
<td>Sigma-Aldrich</td>
<td>5</td>
</tr>
<tr>
<td>N$\omega$-nitro-L-arginine methyl ester (L- NAME)</td>
<td>nitric oxide synthase inhibitor</td>
<td>$10^{-4}$ M</td>
<td>Sigma-Aldrich</td>
<td>6</td>
</tr>
<tr>
<td>NS398</td>
<td>preferential inhibitor of COX2</td>
<td>$10^{-6}$ M</td>
<td>Sigma-Aldrich</td>
<td>7</td>
</tr>
<tr>
<td>Substance</td>
<td>Description</td>
<td>Concentration</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------------------------------------------</td>
<td>----------------</td>
<td>---------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>potassium chloride (analaR grade) (KCL)</td>
<td>VWR International (Poole, England)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S18886</td>
<td>thromboxane prostanoid (TP) receptor antagonist</td>
<td>$10^{-7}$ M</td>
<td>gift from the Institut de Recherches Servier (Suresnes, France)</td>
<td></td>
</tr>
<tr>
<td>SC560</td>
<td>selective inhibitor of COX1</td>
<td>$3 \times 10^{-7}$ M</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>extracellular superoxide scavenger</td>
<td>120 U ml$^{-1}$</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>TRAM 34</td>
<td>Selective blocker of intermediate-conductance calcium activated potassium channels</td>
<td>$10^{-7}$ M</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>UCL 1684</td>
<td>selective blocker of small-conductance calcium activated potassium channels</td>
<td>$10^{-7}$ M</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
</tbody>
</table>

**Genotyping for Lepr$^{db/db}$/TLR4 double mutant mice**

**Materials:**

- DirectPCR-Tail extraction buffer Qiagen
- Protein kinase Invitrogen (Carlsbad, CA, USA)
- Taq Polymerase Fermentas (Ontario, Canada)
- MgCl$_2$ Promega (Fitchburg, WI, USA)
- 10xTag buffer Promega
dNTP  
Promega

QIAquick Gel Extraction Kit  
Qiagen

Ras1 restriction enzyme  
Fermentas

Nla III restriction enzyme  
Fermentas

Custom PCR primers  
Invitrogen

### Primers

1. Leptin receptor: 135 bp amplimer length

   5’ primer sequence: AGA ACG GAC ACT CTT TGA AGT CTC

   3’ primer sequence: CAT TCA AAC CAT AGT TTA GGT TTG TGT

2. TLR4 204 bp amplimer length

   5’ primer sequence: TCA GAA TGA GGA CTG GGT GA

   3’ primer sequence: TCA AAG ATA CAC CAA CGG CTC

### Protocol

1. Ear biopsies were collected and DNA extracted using 100 µl Direct PCR-Tail extraction buffer with protein kinase (0.3 mg/ml) added and incubated at 56°C overnight.
2. Samples were heated at 85°C the following day. To remove undigested materials, they were centrifuged at 12,000 rpm for 5-10 minutes and the supernatant was collected in new microcentrifuge tubes.

3. DNA was used to amplify with leptin receptor primer sets by PCR using Tm at 55°C for 40 cycles.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Per sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Tag buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward Primer 1</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward Primer 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.2</td>
</tr>
<tr>
<td>Water</td>
<td>14.3</td>
</tr>
<tr>
<td>DNA</td>
<td>4</td>
</tr>
</tbody>
</table>

Total 25

4. A restriction enzyme (RE) digestion reaction was set up. Rsa 1 was used to digest the PCR products containing the GTAC sequence, which existed in the mutant leptin receptor gene. Then the reaction mixture was incubated at 37°C overnight.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Per sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 4</td>
<td>2</td>
</tr>
<tr>
<td>Rsa 1</td>
<td>1</td>
</tr>
<tr>
<td>DNA</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

5. RE digestion products were loaded and run in 10% PAGE gel at 160V for 45 minutes.

6. The gel was stained with ethidium bromide containing Tris/Borate/EDTA (TBE) buffer. The gel image was visualised using a Syngene Geldoc image system to determine leptin receptor genotypes. Since the mutant Leptin receptor gene contained a CATG sequence the PCR products were cut with RE, yielding 29 and 108 bps bands, whereas the wild-type genotype was indicated by the undigested products at 135 bps.

7. DNA samples which were Lepr<sup>db/db</sup> were selected. These DNA were amplified with TLR4 primers by PCR using Tm at 55°C for 40 cycles.
8. PCR products were purified using the Qiagen quick Gel Extraction Kit following manufacturer’s instructions.

9. Another restriction enzyme digestion reaction was set up. Nla III was used to digest the PCR products containing CATG sequence. The reaction mixture was incubated at 37°C overnight.
Nla III    1
Water      18
DNA        10

Total      31

10. The RE digestion products were loaded and run in 10% PAGE gel at 160V for 45 minutes.

11. The gel was incubated with ethidium bromide containing TBE. The gel image was visualized using a Syngene Geldoc image system to determine TLR4 genotypes. Mutant TLR4 gene contains a CATG sequence such that PCR products were cut by the RE, yielding 96 and 108 bps bands, whereas wild-type genotype was indicated by the undigested products at 204 bps.
References


Supplement material

Chao-Fan Liang, Jacky TC Liu, Yu Wang, Aimin Xu and Paul M Vanhoutte

Toll-like receptor 4 mutation protects obese mice against endothelial dysfunction by decreasing NADPH oxidase isoforms 1 and 4
Supplementary figure I

Analysis of metabolic parameters in TLR4\textsuperscript{++} and TLR4\textsuperscript{--} mice given standard chow or high fat diet and in Lepr\textsuperscript{dbh/db} fed with standard chow. (A) Food intake and (B) body weight of 12 weeks old mice; Lepr\textsuperscript{dbh/db} were with (open bars, DWM) or without (full bars) TLR4 deletion. (C) Feeding and (D) fasting blood glucose levels of Lepr\textsuperscript{dbh/db} and DWM mice measured biweekly from the fourth to the twelfth week of age. Representative (E) ipGTT and (G) ITT curves at the age of eight weeks. Areas under the curves of (F) ipGTT and (H) ipITT at the age of six, eight and ten weeks. *, P < 0.05 v.s. respective control as indicated; n=4-7.
Cytokine mRNA expression in epididymal fat pads of Lepr\textsuperscript{db/db} and DWM mice.

mRNA expressions of IL1-\(\beta\), IL-6 and TNF\(\alpha\) in epididymal fat revealed by quantitative PCR. Data expressed as fold change normalized to expression levels in Lepr\textsuperscript{db/db} mice preparations. *, P < 0.05 and **, P < 0.01 vs. respective genes from Lepr\textsuperscript{db/db} mice preparations as indicated, n=6.
Supplementary figure III

Arterial blood pressures and aortic vascular responses of Lepr\textsuperscript{db/db} and DWM mice aortae.

(A) Systolic blood pressures were measured by tail cuff plethysmography in 12-week-old TLR4\textsuperscript{+/+} and TLR4\textsuperscript{−/−} mice given either standard chow or high fat diet feeding, and in Lepr\textsuperscript{db/db} mice given standard chow. (B) Cumulative concentration-response curves to sodium nitroprusside obtained during contractions to U46619 (1 to 3 x 10^{-8} M) in aortic rings with endothelium of Lepr\textsuperscript{db/db} and DWM mice at the age of 12 weeks. Data are expressed in percentage of the contractions to U46619. (C) Cumulative concentration-response curves to U46619 were obtained in aortic rings with endothelium from Lepr\textsuperscript{db/db} and DWM mice at the age of 12 weeks. Data are expressed in the percentage of a reference contraction to 60mM KCl.
obtained at the beginning of the experiment. (E) Cumulative concentration-response curves to acetylcholine obtained in the presence of L-NAME (10^{-4} M) during contractions to U46619 in aortic rings with endothelium from Lepr^{db/db} and DWM mice. Data are expressed as percentage of contractions to U46619. *, P < 0.05 vs. respective controls as indicated, n=4-7.
Supplementary figure IV

Effects of removal of endothelium and various inhibitors on EDH-mediated responses in mice mesenteric arteries. (A) Effect of removal of endothelium (-EC), treatment with TRAM 34 ($10^{-7}$ M), UCL 1684 ($10^{-7}$ M) or the combination of TRAM 34 and UCL 1684, and treatment with apocynin ($10^{-4}$ M) on relaxations to acetylcholine [in the presence of L-NAME ($10^{-4}$ M) and indomethacin ($10^{-5}$ M)] in Lepr$^{db/db}$ and DWM mice mesenteric arteries, presented as areas under the curve. *, $P < 0.05$ vs. Lepr$^{db/db}$ with respective treatment; #, $P < 0.05$ vs. control in the mice preparation with respective genotype; n=4-7.
Effects of removal of endothelium and various inhibitors on EDCF-mediated responses in quiescent mice carotid arteries. (A) Effect of removal of endothelium (-EC), or treatment with indomethacin ($5 \times 10^{-6}$ M), SC560 ($3 \times 10^{-7}$ M), NS398 ($10^{-6}$ M), and S18886 ($10^{-7}$ M) on contractions to acetylcholine, in the presence of L-NAME ($10^{-4}$ M), expressed as areas under curves. (B) Contractions to hydrogen peroxide ($H_2O_2$, $10^{-7}$ M to $10^{-4}$ M) in carotid arteries of Lepr<sup>db/db</sup> and DWM mice in the presence of L-NAME expressed as area under the curve. **, $P < 0.05$ vs. Lepr<sup>db/db</sup> mice preparations with respective treatment; #, $P < 0.05$ vs. control in mice preparations with respective genotype; n=4-7.
Effect of LPS on GTT and ITT in TLR4+/+ mice

(A) ipGTT and (B) ipITT curves in eight weeks old mice four hours after the injection of vehicle or LPS. No statistically significant differences were observed; n=4.
Effect of *in vivo* LPS on *ex vivo* acetylcholine-evoked relaxations of mice aortae. (A) Relaxations to acetylcholine during contractions to 1 to 3 x 10^{-8} M U46619, in aortae of wild type and TLR4^{-/-} mice treated with LPS (injection) expressed as areas under the curve. (B) Cumulative concentration-response curves to sodium nitroprusside obtained during contractions to U46619 (1 to 3 x 10^{-8} M) in aortic rings with endothelium from wild type and TLR4^{-/-} mice.
with or without administration of LPS. *, P < 0.05 vs. vehicle or control in wild type mice preparations; #, P < 0.05 vs. LPS treatment in wild type mice preparations; n = 5.

**Supplementary figure VIII**

**Effect of in vivo LPS on ex vivo endothelium-dependent contractions in mice carotid arteries.** (A) Contractions to acetylcholine in the presence of L-NAME (10^{-4} M) in carotid arteries of wild type and TLR4^{−/−} mice treated with LPS (injection) expressed as areas under the curve. (B) Cumulative concentration-contraction curves to U46619 obtained in carotid artery
rings without endothelium from wild type and TLR4<sup>−/−</sup> mice. *, P < 0.05 vs. vehicle or control in wild type mice preparations; #, P < 0.05 vs. LPS treatment in wild type mice preparations; n = 5.

Supplementary figure IX

**Effect of LPS on ROS production in vitro.**

The endothelial cell line EA.hy926, which is derived from human umbilical vein endothelial cells (HUVECs), was grown in Ham's Kaighn's Modification F12K medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 0.1 mg/ml heparin, and 0.05 mg/ml endothelial cell growth supplement. Cells were incubated in 75-cm<sup>2</sup> culture flasks at 37°C in an atmosphere containing 5% CO2-95% room air. The medium was changed every three or four days. For experiments, cells were seeded in six-well plates at a density of 5 x 10<sup>5</sup> cells / well (or at a density of 1.6 x 10<sup>5</sup> cells/well in 12-well plates). Cells were transfected with RNAi specific to
NOX1, NOX4 or scrambled control for 40 hours, followed by stimulation with LPS (10^{-5} M) for eight hours. ROS levels were determined by the lucigenin chemiluminescence assay and expressed as counts/mg. mg protein. LPS-induced superoxide production was significantly decreased by RNAi-mediated knockdown of NOX1 and/or NOX4. *, p<0.05; ** p<0.01, n=5 in each group. Note that the expression of NOX1 and NOX4 in cells transfected with their respective RNAi was decreased by 67% and 72%, respectively, compared to scrambled controls, as determined by real-time PCR (data not shown).