Laboratory Investigation (2008) 88, 1316–1328

Critical role of the NADPH oxidase subunit p47^{phox} on vascular TLR expression and neointimal lesion formation in high-fat diet-induced obesity

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Reactive oxygen species (ROS) formation is associated with inflammation and vasculature dysfunction. We investigated the potential role of the NADPH oxidase on vascular Toll-like receptor (TLR) expression and carotid neointimal formation in high-fat (HF) diet-induced obesity (DIO) model. Using mice DIO and common carotid artery flow cessation-induced lesion formation models, we examined vascular TLR2 and TLR4 expression and neointimal formation in NADPH oxidase subunit p47^{phox}-deficient (p47$^{phox-/-}$) mice. Feeding C57BL/6J mice an HF diet for 22 weeks resulted in significant increases in p47^{phox}, TLR2 and TLR4 expression in vascular tissues compared with mice fed a low-fat (LF) diet. Minimal changes in TLR2 and TLR4 expression was detected in p47$^{phox-/-}$ DIO mice. Furthermore, flow cessation-induced angiogenic and inflammatory response and neointimal formation were significantly attenuated in p47$^{phox-/-}$ DIO mice compared with wild-type DIO mice. In addition, exposure of endothelial cells to leptin led to ROS formation; this was accompanied by upregulation of TLR2, TLR4 expression and its downstream signaling. Leptin also increased endothelial cell migration and proliferation. Pharmacological inhibition of NADPH oxidase or genetic deletion of p47^{phox} significantly diminished these alterations. Obesity increases neointimal formation via a mechanism involving p47^{phox}–TLRs signaling, suggesting that the NADPH oxidase may represent a potential novel therapeutic target for the treatment of obesity-associated vascular inflammation and dysfunction.

Laboratory Investigation (2008) **88,** 1316–1328; doi:10.1038/labinvest.2008.92; published online 8 September 2008

KEYWORDS: leptin; obesity; innate immunity; neointimal formation; reactive oxygen species

Obesity is associated with significantly increased mortality and morbidity from type 2 diabetes and cardiovascular diseases. Although obesity is a well-known independent risk factor for cardiovascular diseases, the mechanisms that relate obesity to vascular endothelial dysfunction is poorly understood. Recently, studies have implicated elevated systemic oxidant stress as an important mechanism linking obesity and the increased risk of atherosclerosis.[1,2] Deletion of the p66*shc* longevity gene to reduce systemic and tissue oxidative stress has been shown to attenuate early atherogenesis in mice fed a high-fat (HF) diet.[3] Furthermore, treatment with an NADPH oxidase inhibitor to reduce reactive oxygen species (ROS) formation has been shown to attenuate the abnormal production of adipocytokines and tumor necrosis factor-α (TNF-α) in adipose tissue.[4]

The multiprotein complex NADPH oxidase, originally identified in phagocytes, is now known to be expressed in endothelial cells. The NADPH oxidases are major sources of ROS in vascular cells and many homologies of the leukocyte NADPH oxidase complex, including p22^{phox}, p47^{phox}, p67^{phox} and gp91^{phox} have been identified in endothelial and vascular smooth muscle cells (VSMCs). Toll-like receptors (TLRs), a family of pattern recognition receptors, is important in the innate immune system. Activation of TLR is linked to the expression of proinflammatory cytokines such as TNF-α and the activation of nuclear factor-κB (NF-κB) signaling pathways in several cell types.[5,6] TLRs are expressed in many cell types including macrophages, adipocytes and skeletal muscle cells. Recently, TLR4 expression has been found in adipocytes isolated from ob/ob and diet-induced obese mouse models, and has been implicated to be involved in the obesity-induced activation of inflammatory and metabolic signaling in insulin resistance.[7–9] TLRs are expressed in the endothelial cells and are upregulated by NADPH

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Received 28 March 2008; revised 15 May 2008; accepted 18 May 2008

oxidase.[10,11] So far, the role of NADPH oxidase on vascular TLR signaling and neointimal formation in obesity has not yet been explored.

Leptin, a protein encoded by the obese (Ob) gene, exerts inflammatory and angiogenic effects on endothelial cells and contributes to vascular dysfunction.[12–15] Obesity is characterized by variable degrees of hyperleptinemia, inflammation and elevated levels of adipocytokines. In addition, leptin has been shown to contribute to the intima–media thickness in obesity.[12–15] A recent study has revealed that treatment with leptin enhances neointimal lesion formation.[16] The role of NADPH oxidase in leptin-induced ROS formation, inflammation and endothelial cell dysfunction are not fully understood.

Using $p47^{phox}$-deficient mice and the HF diet-induced obesity (DIO), we have investigated the essential role of NADPH oxidase on vascular TLR expression and neointimal lesion formation. In addition, we have examined whether leptin-induced vascular ROS formation, inflammation and angiogenesis are dependent on NADPH oxidase.

MATERIALS AND METHODS

Diet-Induced Obesity Model

C57BL/6J mice ($n=30$) were purchased from Jackson Laboratory. The $p47^{phox}$ knockout mice ($n=30$) were originally provided by Dr Steve Holland at NIAID. The $p47^{phox}$ knockout mice (C57BL/6J background) were back breeding for 10 generations. The experimental mice (15 mice/each group) were fed with either a normal chow diet or an HF diet (D12451i 45% kcal diet, Research Diets Inc., NJ, USA) for 22 weeks.

Mouse Common Carotid Ligation Model

Beginning at 22 weeks, mice common carotid artery lesions[17,18] were induced by ligation of the left common carotid artery. At 28 days after surgery, the experimental animals were euthanized to evaluate carotid artery neointimal formation.

Quantification of Neointimal Formation

The carotid arteries were sectioned and stained with hematoxylin and eosin (HE). The neointima and media images were captured at $\times 10$ magnification with a digital camera. Neointima and media areas were measured using Image-Pro Plus software (Media Cybernetics).[14,19]

Neointimal CD31 and ICAM-1 Immunostaining

Carotid arteries were sectioned (10 μm) and incubated with FITC-monoclonal anti-mouse CD31 antibody (1:50; Sigma, MO, USA) or ICAM-1 antibody (1:50; Santa Cruz, CA, USA). Staining for CD31 was examined using confocal microscopy.[20,21] The neointimal neovascularization was expressed as total CD31-positive cells per mm^2. Staining for ICAM-1 was visualized with diaminobenzidine substrate. Sections without primary antibodies were used as control.

Western Immunoblots for $p47^{phox}$, TLR2, TLR4, TNF-α, Ang-2, MyD88 and IRAK-4

Tissue and cell lysates were subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were immunoblotted with anti-$p47^{phox}$ or interleukin-1 receptor-associated kinase-4 (IRAK-4) (1:1000; Cell Signaling Technology, MA, USA), polyclonal rabbit TNF-α, Ang-2 or polyclonal goat TLR2, TLR4, MyD88 (1:1000; Santa Cruz).

Experimental Protocols

Before exposure to 50 ng/ml leptin (recombinant human leptin; R&D System Inc., MN, USA), mouse heart microvascular endothelial cells (MHMECs) were pretreated for 30 min with the NADPH oxidase inhibitors, diphenylene iodinium (DPI, 10 μM; CalBiochem, San Diego, CA, USA) and apocynin (Apo, 200 μM; CalBiochem) or the superoxide dismutase (SOD) mimetic, 4-hydroxy-TEMPO (Tempol, 5 mM; Sigma).

Measurement of NADPH Oxidase Activity and ROS

NADPH oxidase activity was determined by lucigenin-dependent chemiluminescence using a luminometer.[22,23] ROS formation was determined using a probe chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Molecular Probes Inc., OR, USA), as previously described.[22,23]

$p47^{phox}$ Membrane Translocation and Binding to $gp91^{phox}$

For $p47^{phox}$ membrane translocation studies, cell lysates were centrifuged at 10 000 r.p.m. for 10 min. The supernatant was recentrifuged at 100 000 r.p.m. for 24 h. The pellets were centrifuged at 12 000 r.p.m. for 30 min, and the membrane fraction was isolated.[24] The membrane fraction was then detected with anti-$p47^{phox}$ antibody by western blot analysis. For $p47^{phox}$ binding to $gp91^{phox}$ assay, cell lysates were immunoprecipitated with $p47^{phox}$ antibody (2 μg/mg of total cell protein) for 16 h at 4°C, followed by 2 h of incubation with 1:1 protein A:protein G-sepharose. The immunoprecipated lysates were separated using SDS–gel electrophoresis. After transfer to a nitrocellulose membrane, the blot was incubated with anti-mouse $gp91^{phox}$ (1:1000; Santa Cruz) for 1 h. The membrane was washed and incubated with secondary antibody coupled to horseradish peroxidase.

Cell Migration Assay

Migration assays were performed as previously described.[22,23] Polycarbonate filter wells were coated with type I collagen and MHMEC were plated in the upper chamber. The cells were then allowed to migrate for 24 h. Ten randomly selected fields were counted.

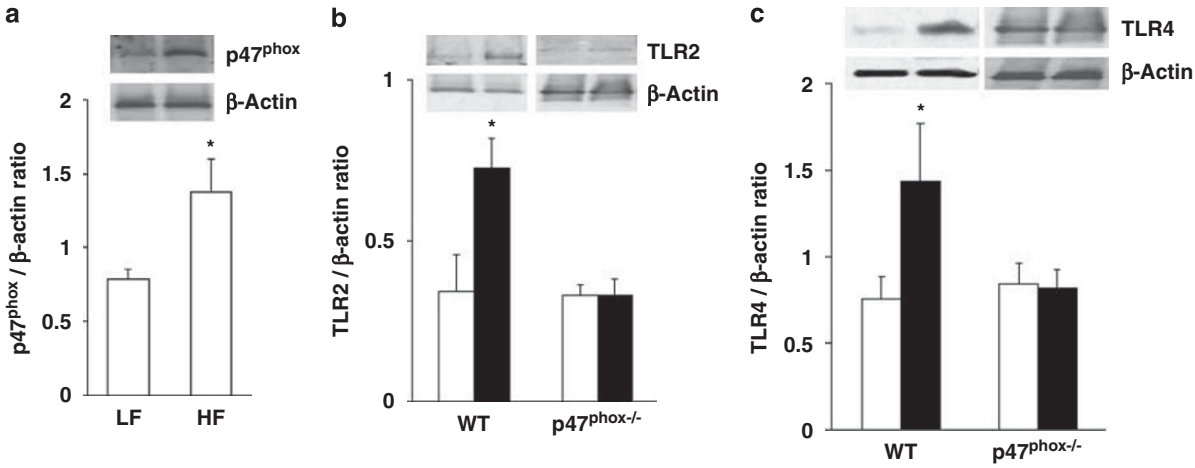


Figure 1 Expression of p47^{phox}, TLR2 and TLR4 in the vascular tissue of the DIO mice. (**a**) Effect of fed a low-fat (LF) or high-fat (HF) diet on p47^{phox} protein expression in the vascular tissues isolated from WT mice. Densitometric data from western blot analyses showing that an HF diet significantly increases p47^{phox} expression in WT DIO mice compared with an LF diet. (**b**) Expression of TLR2 in the vascular tissues of WT or p47^{phox} mice fed an LF or an HF diet. Western blot densitometric data revealing that an HF diet significantly increases TLR2 expression in WT DIO mice (black bars) compared with LF diet mice (white bars), whereas an HF fails to induce TLR2 expression in p47$^{phox-/-}$ mice. (**c**) Expression of TLR4 in the vascular tissues isolated from WT or p47$^{phox-/-}$ mice fed an LF or an HF diet. Densitometric data demonstrating that an HF diet significantly increases TLR4 expression in WT mice fed an HF diet (black bars) compared with LF diet (white bars). An HF had little effect on TLR4 expression in p47$^{phox-/-}$ mice (mean ± s.d., $n = 7$–8 mice, $^*P < 0.05$).

Cell Proliferation by MTT Assay

Cell proliferation was assayed using a cell proliferation (MTT) kit according to the manufacturer's instructions (Roche Diagnostic Corp., IN, USA).

All procedures were in conformance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Statistical Analysis

Statistical analysis was performed using ANOVA followed by *t*-test corrected for multiple comparisons (Student–Newman–Keuls). Significance was set at $P < 0.05$.

RESULTS

p47^{phox} TLR2 and TLR4 Expression in the Vascular Tissue of the DIO Mouse Model

Feeding mice an HF diet for 22 weeks resulted in a dramatic increase in p47^{phox} expression in the vascular tissues of wild-type (WT) DIO mice (Figure 1a). Next, we sought to determine whether an HF diet increases TLR2 and TLR4 expression and whether the absence of p47^{phox} blocks this effect. Feeding mice an HF diet led to significant increases in TLR2 and TLR4 expression in vascular tissues taken from WT DIO mice compared with control mice fed a low-fat (LF) diet (Figure 1b and c). Minimal changes in TLR2 and TLR4 expression were detected in p47$^{phox-/-}$ DIO mice (Figure 1b and c). As shown in Supplementary data 1A and B, feeding WT and p47$^{phox-/-}$ mice an HF diet led to a gradual increase in body weight and fat tissue deposit. Interestingly, the HF-induced body weight gain and fat tissue deposit were less in p47$^{phox-/-}$ DIO mice compared to WT DIO mice.

Deficiency of p47^{phox} Attenuates Carotid Artery Flow Cessation-Induced Neointimal Lesion Formation in the DIO Mouse Model

To investigate the effect of NADPH oxidase on vascular lesion formation, we used carotid artery ligation as a model for neointimal formation in DIO mice. No neointimal lesion was developed in both p47$^{phox-/-}$ and WT carotid arteries after 28 days ligation in the LF-fed mouse model (Figure 2a). A larger neointimal lesion was present in WT DIO mice carotid arteries 28 days after ligation. The neointima was significantly smaller in p47$^{phox-/-}$ DIO carotid arteries (Figure 2b). In addition, neointimal area in the p47$^{phox-/-}$ DIO mice was significantly reduced compared with WT DIO mice (Figure 2c). However, media area had no significant changes both in WT and p47$^{phox-/-}$ DIO mice (data not shown).

Deficiency of p47^{phox} Attenuates Carotid Artery Flow Cessation-Induced Neointimal Angiogenic and Inflammatory Response in the DIO Mouse Model

To determine whether NADPH oxidase promotes neointimal lesion formation via triggering angiogenic and inflammatory cascades, we examined Ang-2 and TNF-α expression in DIO mice. Ang-2 and TNF-α expression were significantly increased in the carotid of WT DIO mice, but not in DIO mice lacking p47^{phox} (Figure 3a and b). Furthermore, our morphological analyses showed a robust inflammatory and angiogenic response in the neointima of WT DIO mice, confirmed by consistent detection of numerous structures positive for an endothelial-specific marker CD31 (Figure 3c) and ICAM-1 (Figure 3d) in the neointima. Importantly, intralesion inflammatory and angiogenic response was

significantly reduced in the p47$^{phox-/-}$ DIO mice (Figure 3c and d).

Expression of Ang-2 and p47^{phox} are Upregulated in Atherosclerotic Neointima of ApoE Deficiency (ApoE KO) Mice

ApoE KO mice fed an HF diet for 24 weeks demonstrated a significant increase in atherosclerotic neointima formation as shown in (Figure 4a and b). Our western blot analysis further demonstrated that p47^{phox} and Ang-2 protein expression in atherosclerotic aorta was upregulated during atherosclerotic lesion progression (Figure 4c).

Leptin Stimulates ROS Formation via Activation of p47^{phox}

Exposure of WT MHMEC to leptin (50 ng/ml) resulted in p47^{phox} membrane translocation and binding to NADPH oxidase membrane subunit gp91^{phox} (Figure 5a and b). Furthermore, exposure of MHMEC to leptin led to an increase in ROS formation (Figure 5c and d). As shown in Figure 5c and d, pretreatment with Apo (200 μM) or the SOD mimetic, tempol (Temp, 5 mM), for 30 min significantly inhibited leptin-induced ROS formation. Deficiency of p47^{phox} abolished leptin-induced ROS formation (Figure 5c).

Leptin-Induced TLR2 and TLR4 Expression is Dependent on the NADPH Oxidase Subunit p47^{phox}

To determine the associations between leptin-induced ROS formation and TLRs signaling, we examined effects of leptin on TLRs expression and its downstream signaling MyD88 and IRAK-4, using p47$^{phox\ +/+}$ and p47$^{phox\ -/-}$ MHMEC. As shown in Figure 6a, exposure of MHMEC to leptin (50 ng/ml) for various time periods results in a gradual increase in TLR2 and TLR4 protein expression seen at 2–4 h. This was accompanied by dramatic increases in MyD88 and IRAK-4 expression (Figure 6b). In p47$^{phox-/-}$ MHMEC, leptin has little effect on TLRs, MyD88 and IRAK-4 protein expression.

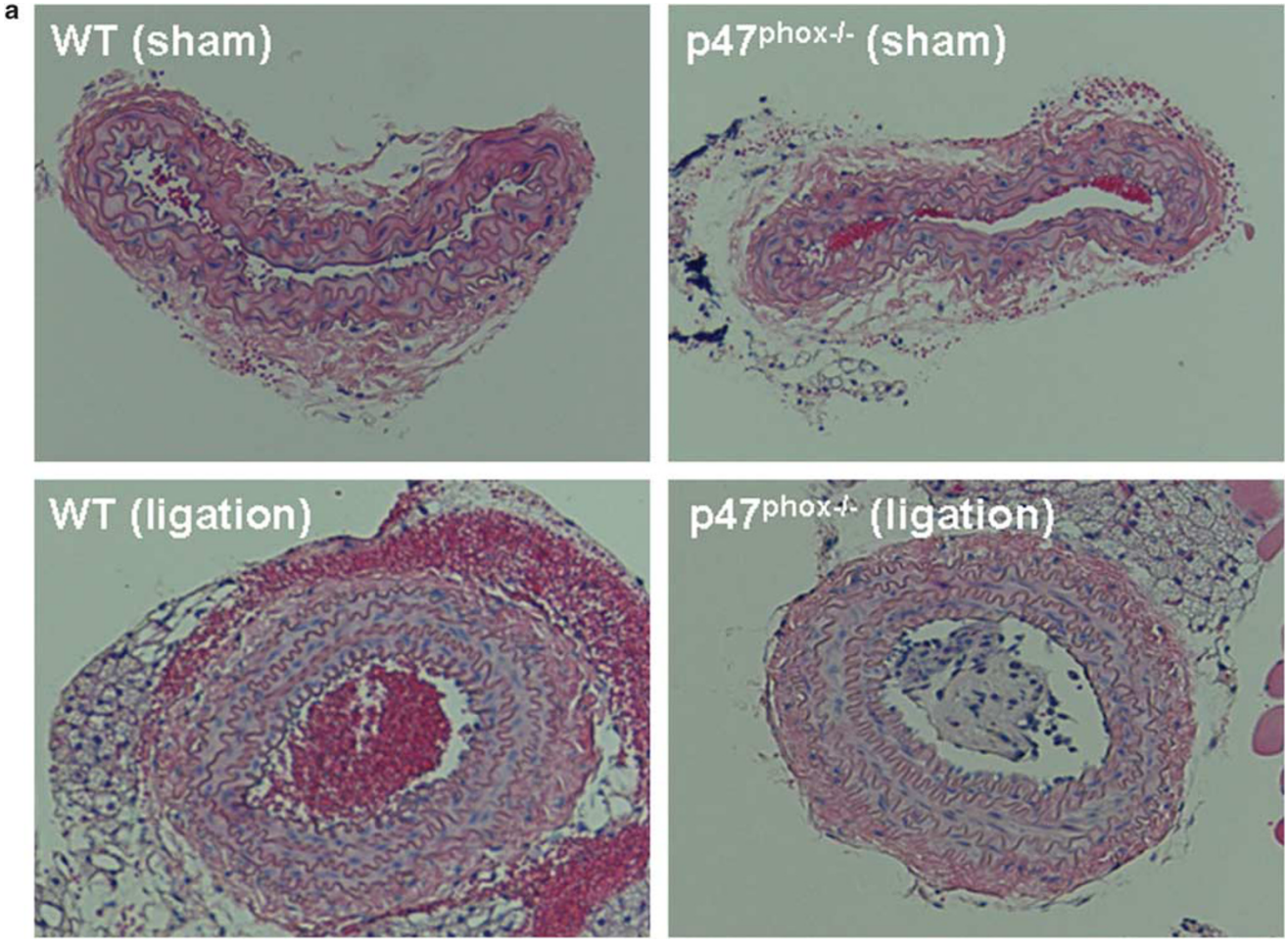
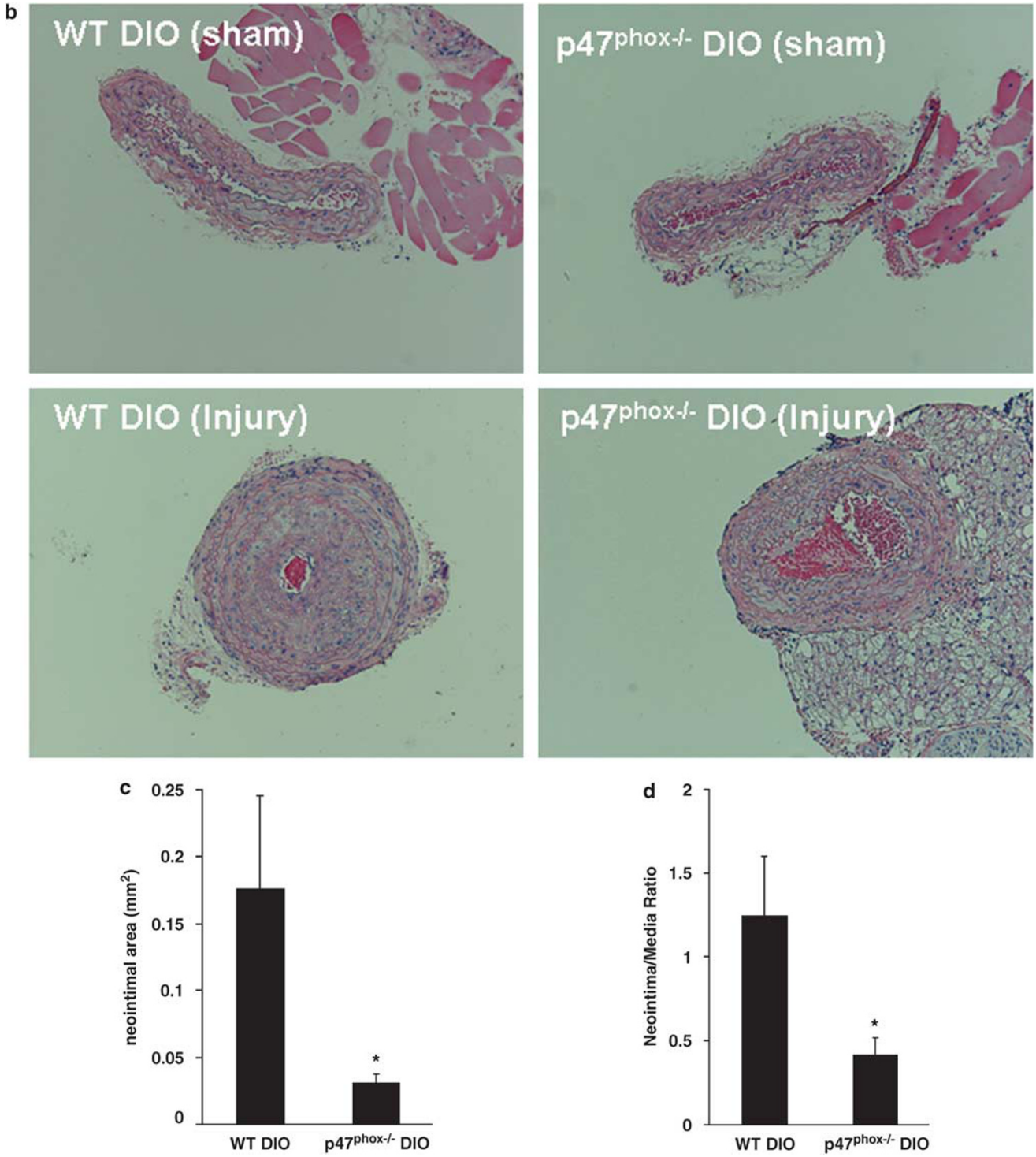


Figure 2 Carotid artery flow cessation-induced neointimal formation in the wild type (WT) and p47$^{phox-/-}$ DIO mice. (**a**) Representative images of cross sections of carotid artery subjected to flow cessation in WT and p47$^{phox-/-}$ mice fed an LF diet. (**b**) Representative cross sections of carotid artery flow cessation-induced neointimal lesion formation in WT and p47$^{phox-/-}$ DIO mice. Cross sections were stained with hematoxylin and eosin (HE). (**c**) Quantitative analysis of neointiamal area in the carotid arteries at 28 days after ligation in DIO mice model. Data are expressed as area of neointimal per mm^2 (mean ± s.d., $n = 7$–8 mice, $^*P < 0.05$, compared with WT DIO mice).



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Inhibition of NADPH Oxidase Attenuates Leptin-Stimulated Endothelial Cell Migration and Proliferation

Exposure of MHMEC to leptin (50 ng/ml) for various time periods up to 24 h resulted in a gradual increase in Ang-2 expression. Exposure of $p47^{phox-/-}$ MHMEC to leptin failed to cause a dramatic induction of Ang-2 expression (Figure 7a). Exposure to leptin resulted in a significant increase in MHMEC migration. Treatment with DPI (10 μM), Apo (200 μM) or Temp (5 mM) significantly attenuated leptin-induced cell migration (Figure 7b). Exposure of MHMEC to

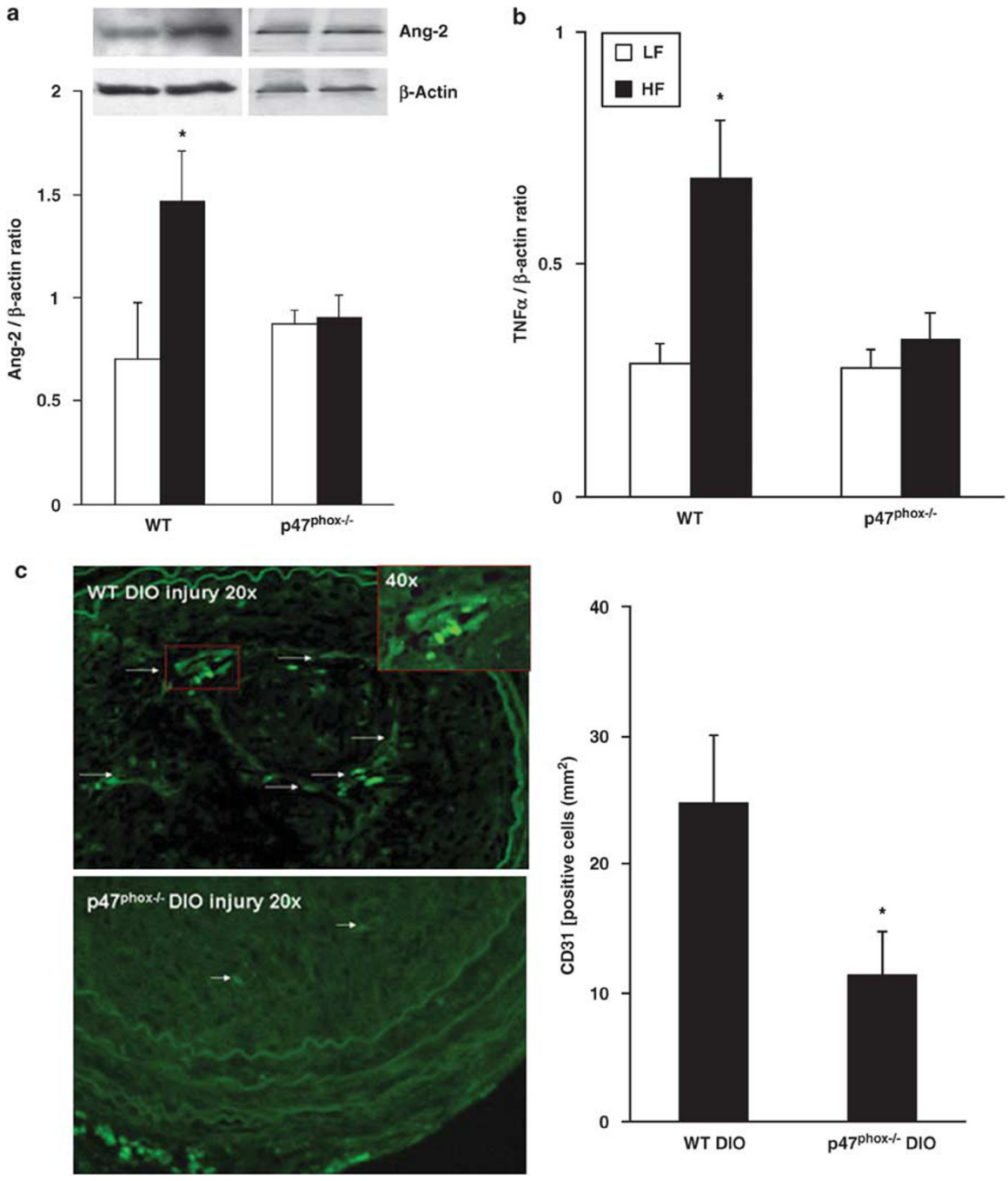

Figure 3 Carotid artery flow cessation-induced neointimal angiogenic and inflammatory response in the wild type (WT) and p47$^{phox-/-}$ DIO mice. (**a**) Representative western blot analysis of Ang-2 expression in the vascular tissue of WT DIO mice and p47$^{phox-/-}$ DIO mice. Densitometric data demonstrating that WT mice fed an HF diet (black bars) results in a significant increase in Ang-2 expression compared with LF diet (white bars), whereas an HF diet fails to induce Ang-2 expression in p47$^{phox-/-}$ mice (mean ± s.d., $n = 7$–8 mice, $^*P < 0.05$). (**b**) Western blot densitometric data demonstrating that WT mice fed an HF diet (black bars) results in a significant increase in TNF-α expression compared with LF diet (white bars), whereas an HF diet fails to induce TNF-α expression in p47$^{phox-/-}$ mice (mean ± s.d., $n = 5$ mice, $^*P < 0.05$). (**c**) (Left) Upper panel: representative cross section of the carotid artery of WT DIO mice at 28 days after surgery staining with the endothelial cell marker, CD31. Upper right inset illustrates a × 40 magnification of one of numerous CD31-positive structures (green, boxed) detected within the neointima (arrows point to some of these). Bottom panel: representative cross section of the carotid artery of p47$^{phox-/-}$ DIO mice staining with CD31. (Right): Quantitative analysis of angiogenic response in the carotid arteries. Data are expressed as total CD31-positive cells per mm^2 (mean ± s.d., $n = 5$ mice, $^*P < 0.05$, compared with WT DIO mice). (**d**) Deficiency of p47^{phox} attenuates carotid artery ligation-induced inflammatory response in DIO mice. (Left) Upper panel: representative cross section of the carotid artery of WT DIO mice at 28 days after surgery staining with ICAM-1. Bottom panel: representative cross section of the carotid artery of p47$^{phox-/-}$ DIO mice staining with ICAM-1. Right: ICAM-1-positive cells in the neointima were significantly reduced in p47$^{phox-/-}$ DIO mice. Data are expressed as total ICAM-1-positive cells per field (10 ×) (mean ± s.d., $n = 5$ mice, $^*P < 0.05$, compared with WT DIO mice).

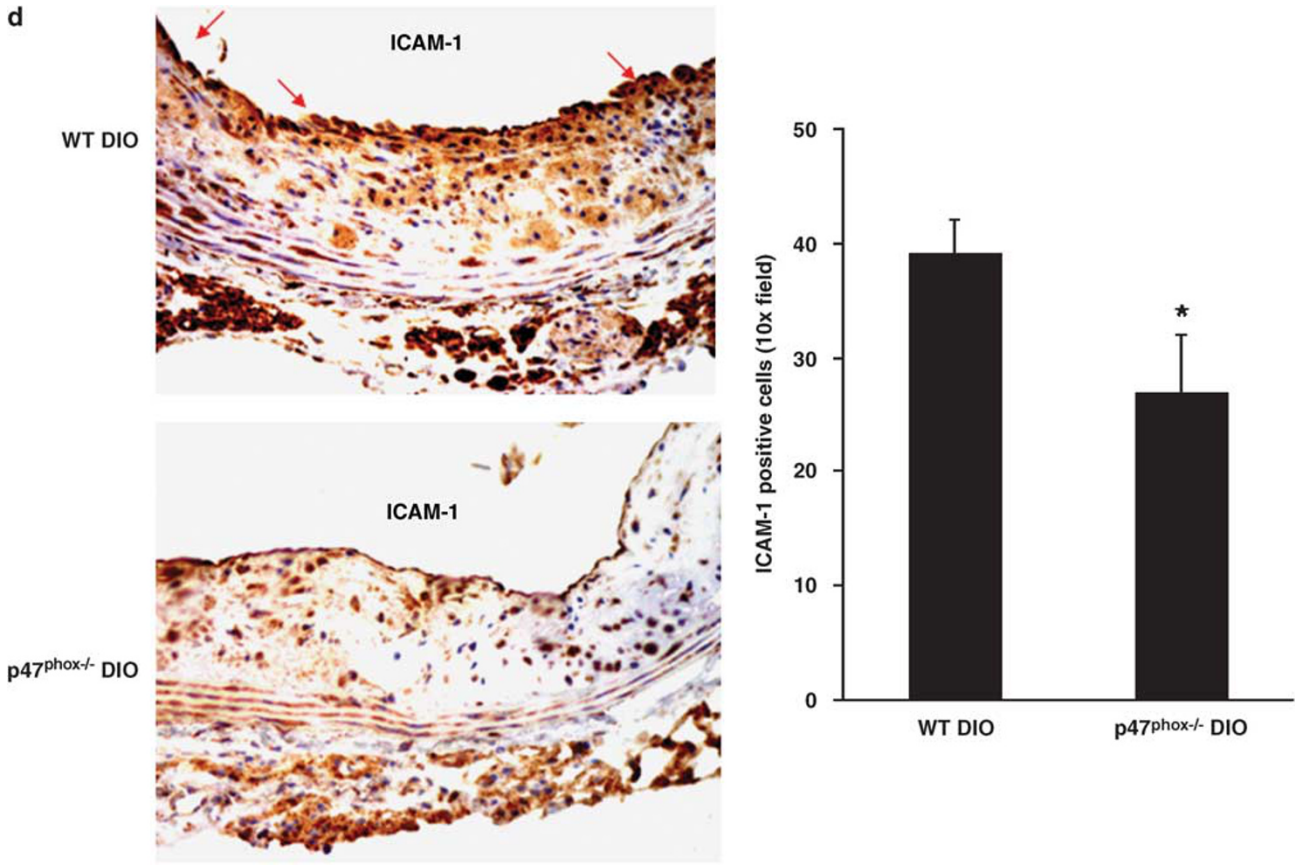

Figure 3 Continued.

leptin for 72 h also significantly increased cell proliferation. Treatment with Apo (200 μM) partially inhibited leptin-induced cell proliferation, whereas DPI (10 μM) or Temp (5 mM) completely abolished leptin-induced cell proliferation (Figure 7c).

Deficiency of the NADPH Oxidase Subunit p47^{phox} on Leptin-Induced Endothelial Cell Migration and Proliferation

Using p47$^{phox-/-}$ MHMEC, we further examined the involvement of p47^{phox} activation on leptin-induced cell migration and proliferation. As shown in Figure 7d, leptin-induced endothelial cell migration was significantly impaired in p47$^{phox-/-}$ MHMEC. Interestingly, deficiency of p47^{phox} completely abolished leptin-induced endothelial cell proliferation (Figure 7e).

DISSCUSSION

In these studies, we have explored the link between obesity and oxidative stress on TLR expression and flow cessation-induced neointimal formation. Our data show that NADPH oxidase and TLR signaling are significantly upregulated in the vascular tissue of DIO mice, and deletion of the NADPH oxidase p47^{phox} abolishes both TLR2 and TLR4 expression in vascular tissue. Deletion of the NADPH oxidase p47^{phox} also blunted flow cessation-induced inflammatory and angiogenic response; this was accompanied by a significant inhibition of neointimal formation in DIO mice. Using p47$^{phox-/-}$ MHMEC, our data demonstrate that leptin upregulates TLRs expression and its downstream signaling via an NADPH oxidase-dependent mechanism. Our studies have provided evidence that NADPH oxidase-derived ROS may be a key component in obesity-induced TLR signaling, vascular inflammation and neointimal formation.

Adipose tissue has been recognized as an active endocrine organ that secretes a variety of proinflammatory cytokines such as leptin, TNF-α and IL-6.[25] Recent studies have demonstrated that DIO also leads to activation of the TLR4 and the NF-κB, which may be involved in obesity-induced inflammation and insulin resistance in adipose tissue.[7–9] Free fatty acid-induced expression of TNF-α and IL-6 is attenuated in the adipocytes of TLR4$^{-/-}$ mice.[7] Our present studies extend these findings and demonstrate that feeding WT mice an HF diet leads to increases in TLR2 and TLR4 expression in the vascular tissue, suggesting that TLR signaling is also a key mediator of inflammation, thereby linking obesity to vascular inflammation and dysfunction.

Clinical studies have implicated elevated oxidant stress as an important mechanism linking obesity and the increased risk of cardiovascular diseases.[1,2] The NADPH oxidase

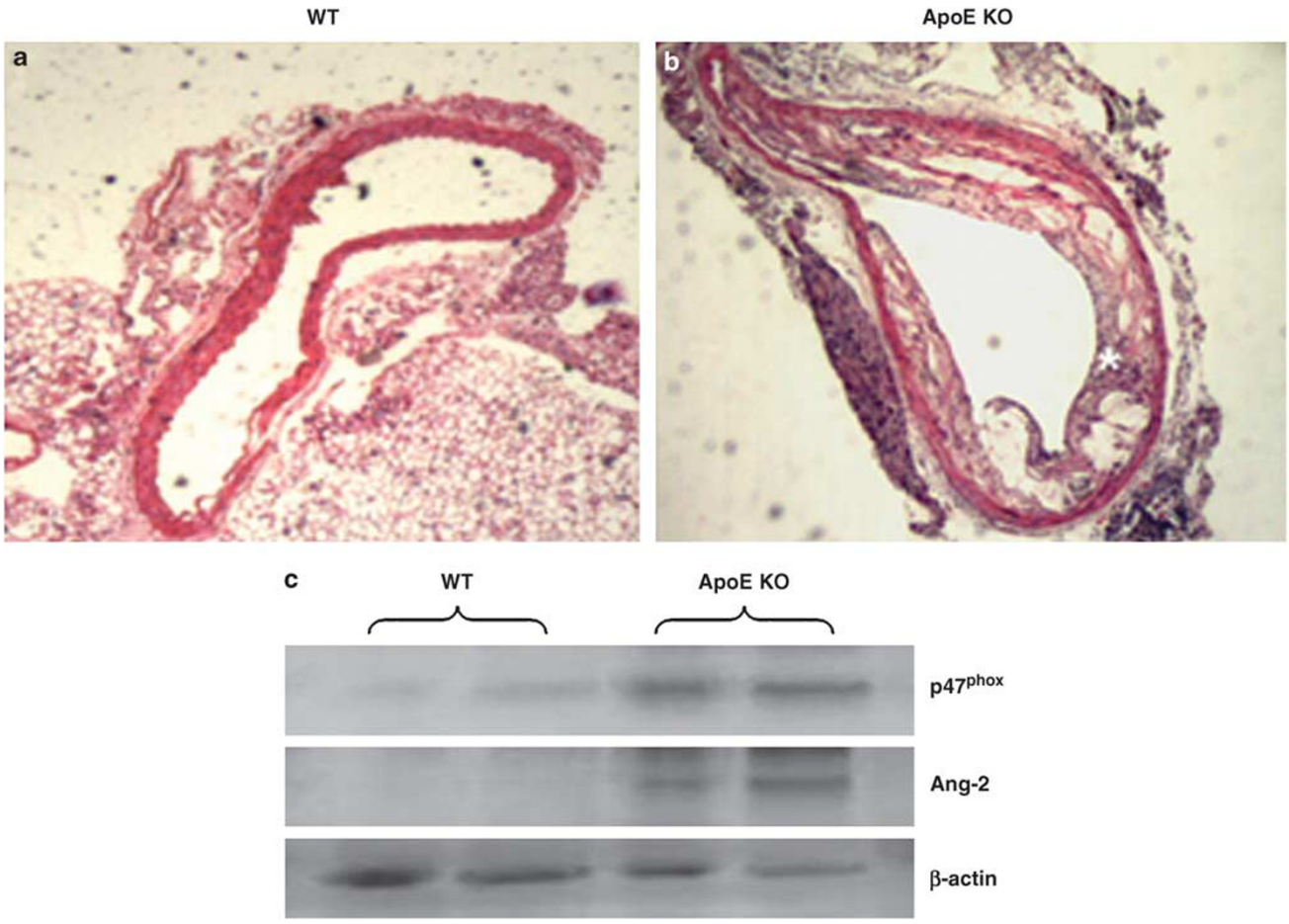

Figure 4 Neointimal formation, Ang-2 and p47^{phox} expression in high fat-diet fed ApoE KO mice. (**a** and **b**) Representative cross sections of aorta atherosclerotic neointimal lesion formation in ApoE deficiency (ApoE KO) mice fed a high-fat diet. Cross sections were stained with hematoxylin and eosin (HE). (**c**) The expression of p47^{phox} and Ang-2 in C57BL/6J (WT) and ApoE (ApoE KO) mouse aorta. p47^{phox} and Ang-2 expressions were increased in the advanced lesion of ApoE$^{-/-}$ mouse aorta.

subunit proteins (gp91^{phox} p22^{phox} and p67^{phox}) are increased in animal models of obesity and in the internal mammary arteries of patients with diabetes[4,26,27] In obese mice, treatment with apocynin reduces ROS production in adipose tissue, attenuates the dysregulation of adipocytokines.[28] Previous studies also implicates that insulin resistance is closely associated with oxidative stress.[29,30] Intake of cream and glucose-stimulated ROS formation is dependent on NADPH oxidase.[32,33] Glucose intake also enhances p47^{phox} expression and increases NF-κB activity.[29] Furthermore, treatment with insulin resulted in a significant suppression of p47^{phox} expression in myocardial infarction patients.[31–33] Our present studies further reveals that feeding WT mice an HF diet leads to increases in p47^{phox} expression. Intriguingly, HF diet-induced TLR2 and TLR4 expression are strikingly attenuated in p47$^{phox-/-}$ mice, implicating that obesity-induced TLR signaling is mediated by a mechanism involving NADPH oxidase. Hyperleptinemia has been shown to be closely associated with obesity, insulin resistance and intima–media thickness. Increased inflammation and oxidative stress have been shown to contribute to the detrimental effects of leptin. So far, the intracellular molecular mechanisms underlying the association between leptin and vascular dysfunction are not fully understood. Our data have revealed that exposure of endothelial cells to a high concentration of leptin ($>$0.5–20 ng/ml normal physiological levels) results in upregulation of TLR signaling, implicating involvement of innate immunity in hyperleptinemia-induced endothelial dysfunction. Our data that deletion of p47phox blunted leptin-induced TLR2 and TLR4 expression and its downstream signaling MyD88 and IRAK-4 expression provide additional intracellular molecular basis between ROS formation and TLR signaling.

Neointimal lesion formation in response to arterial injury is markedly attenuated in NADPH oxidase-deficient mice.[34,35] TLR2 and TLR4 expression are markedly enhanced in human atherosclerotic plaque.[36] Using the ApoE$^{-/-}$ mouse model, it has been shown that triggering TLR2 activation dramatically increases atherosclerotic plaque formation.[37] Furthermore, atherosclerotic plaque formation is significantly reduced in TLR4$^{-/-}$/ApoE$^{-/-}$ mice.[38] TLR2 and TLR4 signaling is involved not only in the development of

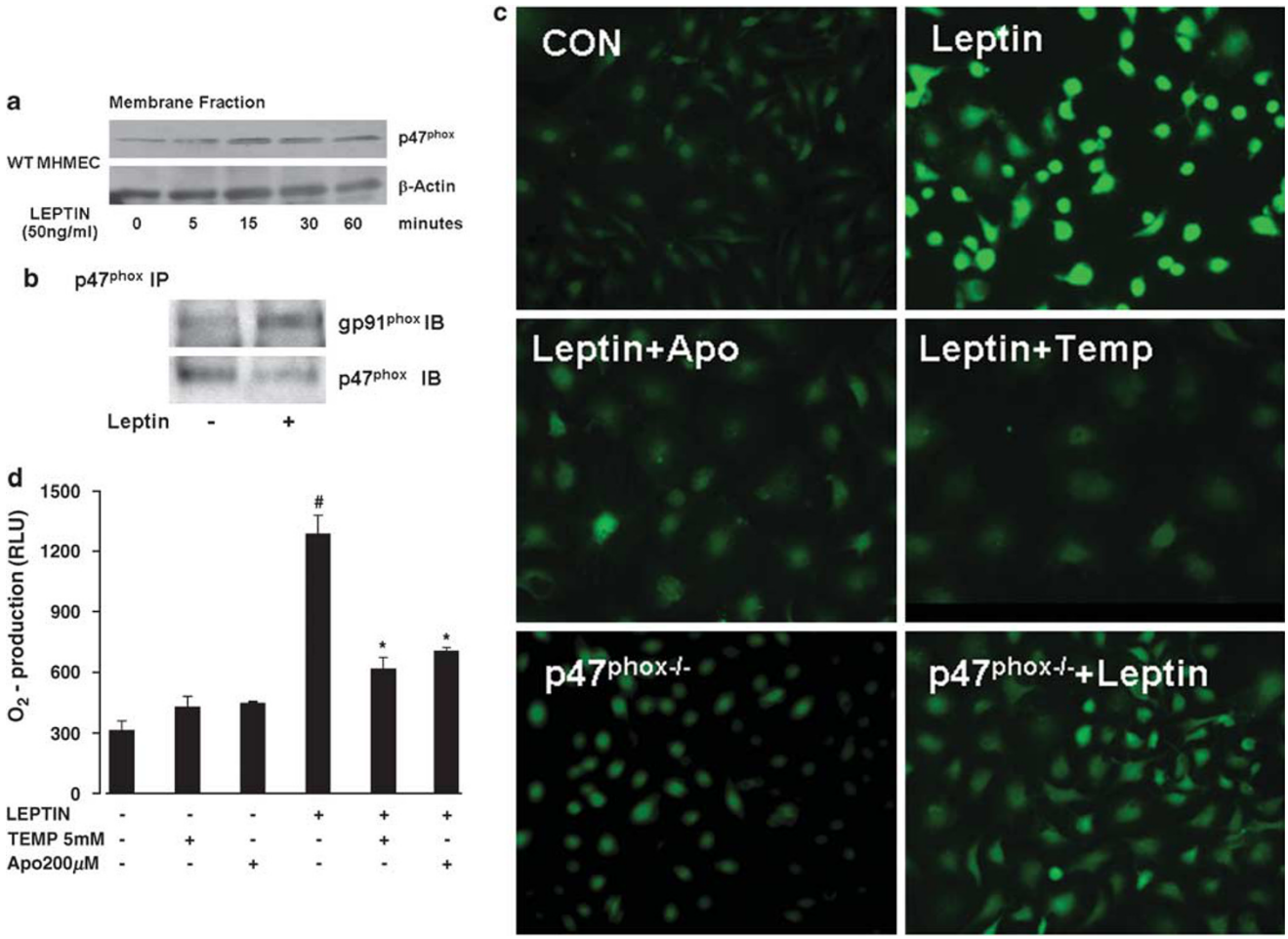

Figure 5 Effect of leptin on NADPH oxidase subunit p47^{phox} activation and ROS formation in microvascular endothelial cells. (**a**) Stimulation of WT MHMEC with leptin for various times up to 60 min caused a gradual increase in p47^{phox} membrane translocation. The figures are representative of an experiment that was repeated at least three times with similar results. (**b**) Stimulation of WT MHMEC with leptin for 30 min resulted in an increase in p47^{phox} binding to gp91^{phox}. The figures are representative of an experiment that was repeated at least three times with similar results. (**c**) Effects of the NADPH oxidase inhibitor, SOD mimetic and deficiency of p47^{phox} on leptin-stimulated ROS formation in MHMEC. Pretreatment of MHMEC with either apocynin (Apo, 200 μM) or the SOD mimetic, tempol (Temp, 5 mM) for 30 min led to suppression of leptin-induced ROS formation. Leptin failed to stimulate ROS formation in p47$^{phox-/-}$ MHMEC. (**d**) Pretreatment of MHMEC with either Apo (200 μM) or Temp (5 mM) for 30 min resulted in significant inhibition of leptin-induced superoxide formation as measured by lucigenin-dependent chemiluminescence. Treatment with Apo or Temp alone had no effect on the basal superoxide production in MHMEC (RLU, relative light units; $n = 3$–4 cell lines, data are mean ± s.d., $^{*}P < 0.05$, compared with leptin; $^{\#}P < 0.05$, compared with baseline).

atherosclerosis but also in neointimal lesion formation. Arterial injury-induced neointimal lesion formation is significantly less in TLR2$^{-/-}$ or TLR4$^{-/-}$ mice.[38–40] Consistent with these findings, our present studies further provide mechanistic data showing that deficiency of the NADPH oxidase subunit p47^{phox} attenuates neointimal lesion formation and TLR expression in DIO mice, suggesting that obesity associated with neointimal lesion formation is mediated by a mechanism involving activation of NADPH oxidase–TLR signaling pathway.

Obesity is associated with increased intima–media thickness of the common carotid artery and that neointimal lesion formation after vascular injury is enhanced in an HF diet-induced animal model of obesity.[14,19] Angiogenesis has likewise been implicated in neointimal formation.[41] Animal models also reveal that angiogenesis is increased after the first 4 weeks of an HF diet.[42] Treatment with the angiogenesis inhibitors prevents lesion progression in ApoE$^{-/-}$ mice.[21] In contrast, treatment with VEGF stimulates plaque angiogenesis and promotes lesion progression both in ApoE$^{-/-}$ mice and in cholesterol-fed rabbit models.[43] There is a notable dearth of knowledge about the signaling processes that link ROS, angiogenesis and neointimal lesion formation. For instance, overexpression of the NADPH oxidase subunit, p22^{phox} has been shown to increase HIF-1α, VEGF expression and intralesional angiogenesis and to promote experimental atheroma progression.[44] Our present data demonstrated that deficiency of the NADPH oxidase p47^{phox} attenuates Ang-2 expression and blunts angiogenic response in the neointima of DIO mice. Our data also show that leptin-induced Ang-2

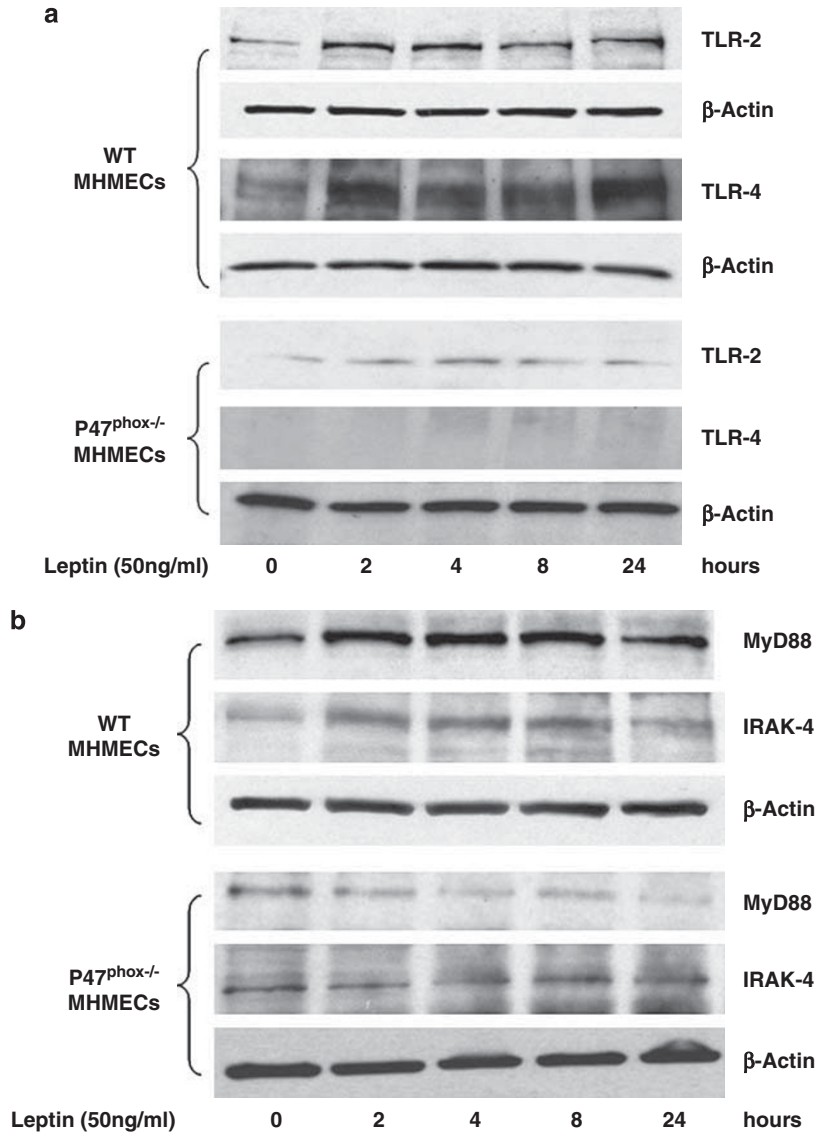

Figure 6 Deficiency of $p47^{phox}$ attenuates leptin-induced TLR2, TLR4 expression and its downstream signaling. (**a**) Exposure of WT MHMEC to leptin for various times up to 24 h led to an increase in TLR2 and TLR4 expression seen at 2–4 h. Leptin failed to induce TLR2 expression in the $p47^{phox-/-}$ MHMEC. TLR4 protein was undetectable in the $p47^{phox-/-}$ MHMEC. (**b**) Exposure of WT MHMEC to leptin for various times up to 24 h increased TLRs downstream signaling MyD88 and IRAK-4 expression, whereas leptin failed to induce MyD88 and IRAK-4 expression in the $p47^{phox-/-}$MHMEC. The figures are representative of an experiment that was repeated at least three times with similar results.

expression, as well as cell migration and proliferation are dependent on NADPH oxidase. These data strongly suggest that NADPH oxidase is a key trigger of neointimal angiogenesis and is essential for the lesion formation in the setting of obesity. In addition, our *in vivo* studies reveal that neointimal ICAM-1 and TNF-α expression is significantly reduced in $p47^{phox-/-}$ DIO, suggesting a role of inflammation in obesity-induced neointimal formation. Although our present studies demonstrated that carotid ligation induced a significant neointimal formation in DIO mice, we did not observe neointimal formation in control mice as other investigator reported.[17,18] Different mouse strain and genetic background have been shown to have a significant different in the neointimal formation in carotid ligation model.[45,46] Whether these factors contribute to this discrepancy in present studies remains unknown. In addition, fed ApoE KO mice with HF diet resulted in a significant atherosclerotic neointimal formation and this was accompanied by dramatic increases in $p47^{phox}$ and Ang-2 expression.

Although elevated leptin concentrations are associated with the intima–media thickness of the common carotid artery in obese patients and that treatment with leptin promotes neointimal formation,[14–16] our present studies did not detect the correlation between the circulating leptin levels with neointimal thickening in DIO mice model. In addition, the metabolic intracellular mechanisms by which DIO modulates NADPH oxidase activation and ROS formation, whether free fatty acids and insulin

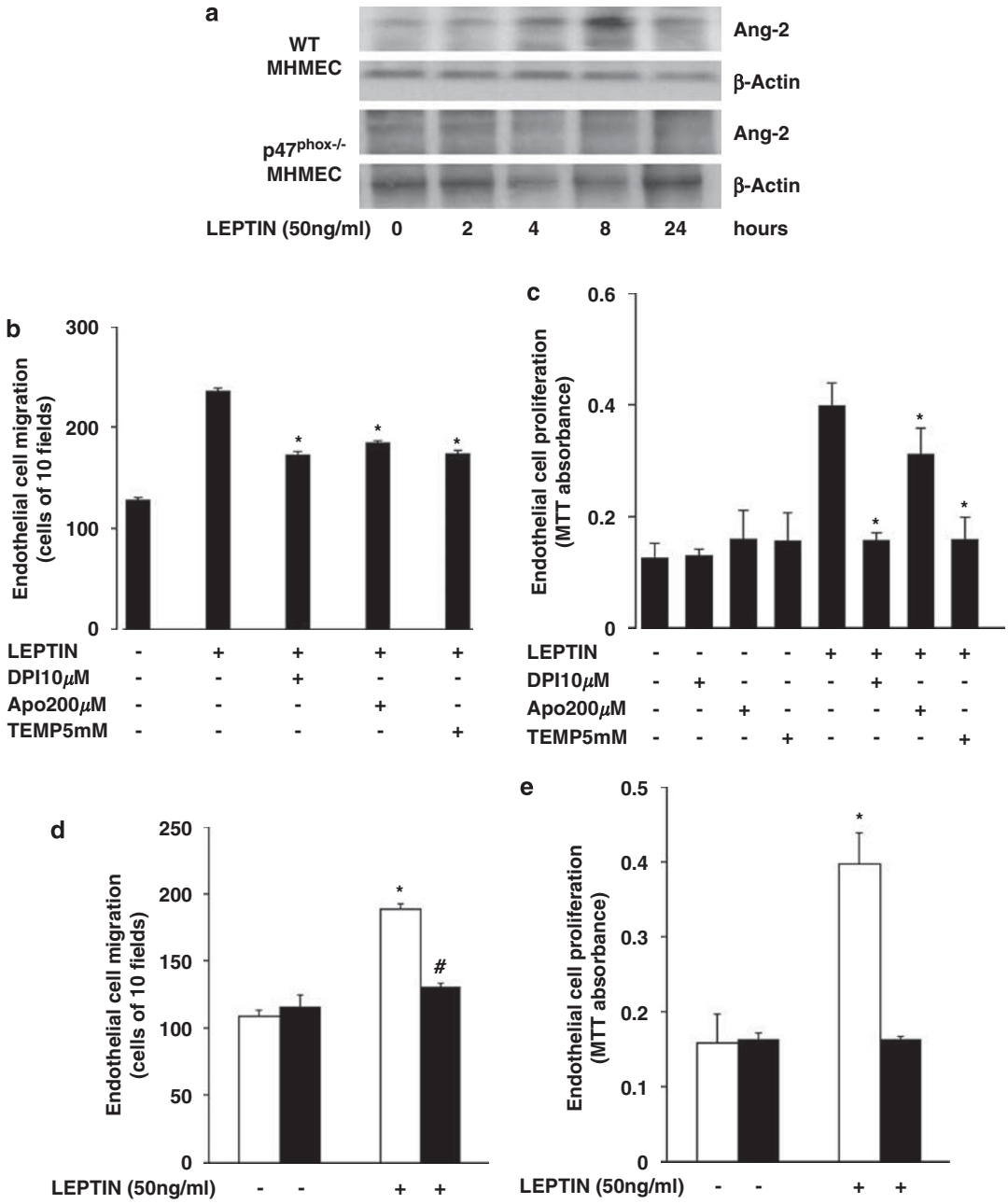

Figure 7 Pharmacological inhibition or genetic deletion of NADPH oxidase on leptin-stimulated endothelial cell migration and proliferation. (**a**) Exposure of WT MHMEC to leptin for various times up to 24 h increased Ang-2 expression, whereas leptin failed to induce Ang-2 expression in the $p47^{phox-/-}$ MHMEC. The figures are representative of an experiment that was repeated at least three times with similar results. (**b**) Treatment of MHMEC with the NADPH oxidase inhibitors, diphenylene iodinium (DPI, 10 μM) and Apo (200 μM), or the SOD mimetic, Temp (5 mM), resulted in significant inhibition of leptin-induced endothelial cell migration as measured by Boyden chamber assay ($n=3$ cell lines, data are mean ± s.d., $^*P<0.05$, when compared with leptin). (**c**) Treatment of MHMEC with DPI (10 μM) and Apo (200 μM) or Temp (5 mM) resulted in significant inhibition of leptin-induced endothelial cell proliferation as measured by MTT assay. Treatment with DPI, Apo or Temp alone had little effect on cell proliferation ($n=4$ cell lines, data are mean ± s.d., $^*P<0.05$, compared with leptin). (**d**) Deficiency of $p47^{phox}$ attenuates leptin-induced endothelial cell migration. Leptin-induced cell migration was significantly reduced in $p47^{phox-/-}$ MHMEC (black bars) compared with $p47^{phox+/+}$ MHMEC (white bars, $n=3$ cell lines, data are mean ± s.d., $^*P<0.05$, compared with $p47^{phox+/+}$ MHMEC, $^{\#}P<0.05$, compared with $p47^{phox+/+}$ MHMEC stimulated with leptin). (**e**) Deficiency of $p47^{phox}$ abolished leptin-induced endothelial cell proliferation. Leptin had little effect on the cell proliferation in $p47^{phox-/-}$ MHMEC (black bars) compared with $p47^{phox+/+}$ MHMEC (white bars, $n=4$ cell lines, data are mean ± s.d., $^*P<0.05$, compared with $p47^{phox+/+}$ MHMEC).

resistance in DIO is responsible for these alterations remain unknown. Further studies are warranted to links these missing.

In summary, we have shown that HF DIO activates NADPH oxidase and mediates the expression of TLR in the vascular tissues. Deficiency of the NADPH oxidase subunit

$p47^{phox}$ attenuates carotid arterial flow cessation-induced angiogenic, inflammatory response and neointimal formation in DIO mice. Our studies elucidate the importance of NADPH oxidase in obesity-induced TLR signaling and inflammation. Pharmacologic or genetic manipulation of the NADPH oxidase system may represent a novel strategy in the treatment of obesity-associated cardiovascular disease.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENT

This work was supported by grants from the American Heart Association grant 0565196B and NIH grant DK074995 (to JX Chen).

DISCLOSURE

All authors declared no conflict of interests.

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