Nox4 is a Novel Inducible Source of Reactive Oxygen Species in Monocytes and Macrophages and Mediates Oxidized Low Density Lipoprotein–Induced Macrophage Death

Chi Fung Lee, Mu Qiao, Katrin Schröder, Qingwei Zhao, Reto Asmis

Rationale: The enhanced formation of intracellular reactive oxygen species (ROS) induced by oxidized low-density lipoprotein (OxLDL) promotes macrophage death, a process likely to contribute to the formation of necrotic cores and the progression of atherosclerotic lesions. Yet macrophage deficiency of phagocytic NADPH oxidase (Nox2), the primary source of ROS in macrophages, does not reduce atherosclerotic lesion development in mice. This suggests an as yet unidentified NADPH oxidase may be present in macrophages and responsible for the intracellular ROS formation induced by OxLDL.

Objective: The aim of this study was to identify the source of intracellular ROS involved in macrophage death.

Methods and Results: Nox4 was expressed in human monocytes and mature macrophages, and was localized to the endoplasmic reticulum and to defined foci within the nucleus. Nox4 colocalized with p22phox, and both proteins were upregulated in response to OxLDL stimulation, whereas Nox2/gp91phox levels remained unchanged. Induction of Nox4 expression, intracellular ROS formation and macrophage cytotoxicity induced by OxLDL were blocked by MEK1/2 inhibition, but not by inhibitors of p38-MAPK (mitogen-activated protein kinase), JNK (Jun N-terminal kinase), or JAK2 (Janus kinase 2). Small interfering RNA knockdown of Nox4 inhibited both intracellular ROS production and macrophage cytotoxicity induced by OxLDL, whereas Nox4 overexpression enhanced both OxLDL-stimulated ROS formation and macrophage death.

Conclusions: Nox4 is a novel source of intracellular ROS in human monocytes and macrophages. Induction of Nox4 by OxLDL is mediated by the MEK1/ERK pathway and required for OxLDL cytotoxicity in human macrophages, implicating monocytic Nox4 in atherogenesis. (Circ Res. 2010;106:1489-1497.)

Key Words: NADPH oxidase n macrophage death n reactive oxygen species n redox signaling n atherosclerosis

Hydrogen peroxide, superoxide anion, and other reactive oxygen species (ROS) have long been considered toxic by-products of metabolic processes and mediators of oxidative stress. Only recently has the essential role of ROS in cell signaling and cell function been recognized.1-3 Although ROS are generated by a variety of cellular sources including mitochondria, cytochrome P450 enzymes and the uncoupling of NOS, these ROS are byproducts and not the primary products of these systems. In contrast, NADPH oxidases (Nox) are professional ROS producers. For example, Nox2/gp91phox, the prototype of NADPH oxidases, is responsible for the oxidative burst in neutrophils and macrophages required for bacterial killing.4,5 The discovery of Nox2 homologues including Nox1, -3, -4, and more distantly related Nox5, Duox1 and Duox2 in numerous different tissues suggests a much broader role for Nox enzymes and the ROS they generate.6,7 For example, Nox4-dependent ROS production is activated in response to an array of extracellular stimuli, including bone morphogenetic protein 48 and transforming growth factor-β9,10 and mediates a number of different biological functions, such as angiogenesis,11 cell adhesion,9 cell migration,12 and vascular remodeling.13 However, to date, only a limited number of targets of these signaling ROS have been identified and validated as bona fide targets of ROS signaling, with reactive cysteine residues of protein tyrosine phosphatases being one of the best-established.

Oxidized low-density lipoprotein (OxLDL) is an important biomarker of cardiovascular diseases. OxLDL levels are elevated in patients with chronic metabolic disorders,14,15 and in atherosclerosis-prone apolipoprotein E–null mice, Itabe and colleagues demonstrated a transient increase in circulating OxLDL levels during the progression of atherosclerosis.16 OxLDL is found in atherosclerotic lesions and one of its

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many proatherogenic properties is its cytotoxicity toward all vascular cells and macrophages. Enhanced macrophage death in atherosclerotic lesions promotes the formation of necrotic cores, a characteristic feature of advanced atherosclerotic plaques. Increased intracellular ROS formation plays a critical role in OxLDL-induced macrophage death but the source of these intracellular ROS has not been known. Nox2 is the primary source of superoxide in macrophages, but Nox2 deficiency in macrophages does not reduce atherosclerotic lesion development in mice. Nox2 activity is regulated by various cytosolic factors, but the source generated by the Nox2 system are either directed to extracellular or to the phagosomes; they are therefore not a likely source of the intracellular ROS observed in macrophages in response to OxLDL stimulation. We thus hypothesized that an as yet unidentified NADPH oxidase may be present in macrophages and is responsible for intracellular ROS formation induced by OxLDL. In the present study, we now provide the first evidence for the existence of a second NADPH oxidase, Nox4, in human monocytes and macrophages. We show that Nox4 is localized in intracellular compartments and colocalizes with its dimerization partner, p22phox. We also provide evidence that OxLDL, but not native LDL, induces the expression of Nox4/p22phox system, and that Nox4, not Nox2, is the source of OxLDL-induced intracellular ROS responsible for macrophage death.

## Methods

### Materials

All chemicals were obtained from Sigma (St Louis, Mo) unless stated otherwise. PDI antibody was purchased from BD Biosciences. p22phox, β-actin, and Nox2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), and the Lamp-1 antibody from Abcam (Cambridge, Mass). Secondary anti-mouse IgG Cy3 and anti-rabbit IgG Cy5 antibodies were purchased from Jackson ImmunoResearch (West Grove, Pa). Protein G Sepharose was purchased from GE Healthcare (Piscataway, NJ).

### Human Monocyte-Derived Macrophage Isolation and Culture

Mononuclear cells were isolated from blood obtained from healthy donors (South Texas Blood and Tissue Center) and mature human mononuclear cells were isolated from blood obtained from healthy donors (South Texas Blood and Tissue Center) and mature human mononuclear cells were isolated from blood obtained from healthy donors (South Texas Blood and Tissue Center). All chemicals were obtained from Sigma (St Louis, Mo) unless stated otherwise. PDI antibody was purchased from BD Biosciences. All chemicals were obtained from Sigma (St Louis, Mo) unless stated otherwise. PDI antibody was purchased from BD Biosciences. All chemicals were obtained from Sigma (St Louis, Mo) unless stated otherwise. PDI antibody was purchased from BD Biosciences. All chemicals were obtained from Sigma (St Louis, Mo) unless stated otherwise. PDI antibody was purchased from BD Biosciences.
Subcellular Fractionation
Subcellular fractionation and isolation of macrophage nuclei was performed according the protocol described Lorenzen et al.30 HMDMs were washed with PBS and lysed with fractionation buffer (20 mmol/L HEPES, pH 7.4, 250 mmol/L sucrose, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1.5 mmol/L EDTA with protease inhibitor [Roche]). Cell lysates were passed through 25G needles 10 times with syringe. The lysates were then left on ice for 20 minutes and centrifuged at 720g for 5 minutes. The pellet representing the nuclear fraction was washed twice with fractionation buffer, passed through 25-gauge needles 10 times, pelleted at 720g for 5 minutes, and subjected to Western blot analysis.

Intracellular ROS and Cytotoxicity Assays
HMDMs were loaded with dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) and 3H-labeled adenine (GE Healthcare) for 2 hour, washed to remove excess dye and radiolabel, and subsequently, stimulated with LDL or OxLDL. Intracellular ROS formation and cytotoxicity were measured as described previously.18,19

Adenovirus Generation
Doxycycline-inducible adenoviruses expressing human Nox4 under the control of a tet-on promoter construct were generated using the pAdEasy system as described elsewhere.31 The cDNA for human Nox4 was kindly provided by Dr Lena Serrander.32 Adenoviruses expressing Nox4 small interfering (si)RNA and control-siRNA were kind gifts from Dr Kai Chen.33 All infections were performed at 50 MOI.

Statistical Analysis
Results are expressed as means±SD of at least 3 independent experiments. Data were analyzed either by paired 2-tailed t test or by ANOVA with the Tukey post hoc test for multi-group comparisons where appropriate. A probability value of less than 0.05 was considered statistically significant.

Results
Human Monocytes and HMDMs Express Nox4
To search for new Nox isoforms that may be responsible for OxLDL-induced intracellular ROS formation in macrophages we performed expression profiling by RT-PCR for Nox1 through 5. In addition to Nox2, freshly isolated, adhesion-purified human monocytes and 2-week-old mature and fully differentiated HMDMs expressed Nox4, but we did not detect mRNA for Nox1, 3 or 5 in either monocytes or HMDMs (Figure 1). Importantly, the treatment of HMDMs with OxLDL induced Nox4 mRNA levels up to 4.6-fold, indicating that Nox4 may be the source of OxLDL-induced ROS formation. Western blot analysis of HMDM lysates with commercially available antibodies directed against Nox4 failed to demonstrate a band at 66 kDa, which would correspond to full-length Nox4 protein. We therefore generated new rabbit polyclonal and rabbit monoclonal antibodies directed against a region in the Nox4 NADPH binding domain. The polyclonal and monoclonal antibodies detected endogenous Nox4 proteins in both human and murine cells at the predicted molecular weight (Online Figure I). Using lysates from both Nox4 overexpressing cells and cells from Nox4 knockout mice, we confirmed the specificity of both the rabbit polyclonal (not shown) and monoclonal Nox4 antibodies (Online Figure I).

Endogenous Macrophage Nox4 Localizes to the Endoplasmic Reticulum and the Nucleus and Colocalizes With p22phox
The minimal requirement for Nox4 activity appears to be the dimerization with p22phox.34,35 Optical sectioning of HMDMs stained with fluorescently labeled anti-Nox4 monoclonal antibodies revealed within the macrophages a high degree of colocalization of Nox4 with its dimerization partner p22phox (Figure 2A). We also found coimmunoprecipitation of p22phox with Nox4, suggesting that endogenous Nox4 in macrophages may indeed be active and account for at least some of the intracellular ROS we detected in resting HMDMs.18

Nox4 protein also colocalized with PDI, an endoplasmic reticulum marker (Figure 2B), confirming similar findings reported by Chen et al for human aortic endothelial cells.33 However, Nox4 staining was not restricted to the ER, indicating that Nox4 also localizes to other intracellular sites within macrophages. For example, we observed Nox4 staining associated with discrete foci within macrophage nuclei (Figure 2C). Z-plane sectioning confirmed that these Nox4-positive foci are located inside the nucleus (not shown). The presence of Nox4 within macrophage nuclei was confirmed by Western blot analysis of nuclear lysates generated by cell fractionation of HMDMs (Online Figure II).

To examine whether Nox4 is expressed by macrophages found in atherosclerotic lesions, we performed immunofluorescence studies in aortic root sections from dyslipidemic LDL-receptor deficient mice. Although a significant fraction of Nox4 staining did not overlap with the macrophage-specific marker CD68, we observed significant colocalization of CD68 with Nox4, suggesting that macrophages in atherosclerotic lesion express Nox4 (Online Figure III).
OxLDL Induces the Concomitant Expression of Nox4 and p22phox

Previously, we showed that OxLDL induces intracellular ROS formation and promotes macrophage death. Because OxLDL stimulation increases Nox4 mRNA levels (Figure 3A) but not Nox2 mRNA in HMDMs (Figure 3B), we next examined whether Nox4 is the source of the intracellular ROS responsible for OxLDL cytotoxicity. However, to have Nox4 account for OxLDL-induced ROS formation would require the upregulation of both Nox4 and p22phox by OxLDL. Stimulation of HMDMs with increasing concentrations of OxLDL increased Nox4 mRNA and protein levels (Figure 3A and 3C), suggesting that Nox4 activity is indeed transcriptionally regulated in macrophages as has been reported for other cell types, rather than being regulated by cytosolic factors as with Nox2. Importantly, OxLDL-induced increases in Nox4 protein levels were paralleled by increased p22phox protein expression, both reaching maximal expression levels after 6 hours (Figure 3D). In contrast, Nox2 protein expression levels in HMDMs remained unchanged in response to OxLDL (Online Figure IV, A). Because p22phox is the obligate dimerization partner required for Nox4 activity, OxLDL appears to induce both components necessary for increased ROS formation, suggesting a role for Nox4/p22phox in OxLDL-induced macrophage death.

Native LDL did not induce Nox4 expression in HMDMs (Online Figure IV, B), implicating lipid and/or protein oxidation products of OxLDL in the induction of the Nox4/p22phox complex. This finding is consistent with previous studies, demonstrating that native LDL neither induces ROS formation nor promotes cytotoxicity in HMDMs. Trolox, a peroxyl radical scavenger, protects HMDMs from OxLDL-induced cytotoxicity, but did not block OxLDL-induced Nox4 upregulation (Online Figure IV, C and D). This suggests that the transcriptional activation of Nox4 does not appear to be mediated by peroxyl radicals, which are generated both during the oxidation of LDL and the breakdown of lipid peroxides within OxLDL. This finding is also consistent with a role for Nox4 upstream of intracellular ROS production and cytotoxicity induced by OxLDL.

Figure 2. Nox4 protein colocalizes with p22phox and is localized in the endoplasmic reticulum and the nucleus of HMDM. Immunohistochemistry and confocal microscopy was performed on HMDMs stained with our new rabbit monoclonal antibody directed against Nox4 (red) and p22phox (green) (A) or the ER-specific marker PDI (green) (B) or DAPI (blue; arrows identify discrete Nox4 loci) (C). The overlays show the extent of colocalization (yellow) of Nox4 with each of these markers. Bar: 10 μm. D, HMDM lysates were prepared and Nox4 protein was immunoprecipitated as described in Methods. Immunoprecipitates were subjected to Western blot analysis and probed for Nox4 and p22phox.
Inhibition of MEK Prevents Nox4 Expression, Intracellular ROS Formation, and Cytotoxicity Induced by OxLDL in HMDMs

To gain insights into the mechanisms involved in OxLDL-induced Nox4 expression and its potential role in macrophage death, we examined the effect of different kinase inhibitors on OxLDL-stimulated intracellular ROS formation and cytotoxicity. HMDMs were preincubated with the MEK1/2 (MAPK/extracellular signal-regulated kinase kinase 1/2) inhibitor U0126, the p38-MAPK (mitogen-activated protein kinase) inhibitor SB203580, or the Jun N-terminal kinase (JNK) inhibitor SP600125, and were subsequently stimulated with 75 μg/mL of OxLDL. Inhibition of MEK1/2 by U0126 completely blocked OxLDL-induced Nox4 expression, whereas neither p38-MAPK nor JNK or Janus kinase (JAK)2 inhibitors showed any effect on Nox4 expression (Figure 4A and 4B; Online Figure V, A). A second MEK1/2 inhibitor, PD98059, showed inhibitory effects very similar to U0126 (not shown), confirming the role of MEK1/2 in the OxLDL-induced upregulation of Nox4. Inhibition of MEK also blocked p22^phox expression induced by OxLDL, whereas Nox2 protein levels were unaffected by MEK inhibition.
MEK1/2 inhibitors not only blocked OxLDL-stimulated Nox4 expression, they inhibited intracellular ROS formation and macrophage death induced by OxLDL. In contrast, p38-MAPK, JNK, and JAK2 inhibitors showed no protective effects (Figure 4C and 4D; Online Figure V, B). These results further support our hypothesis that OxLDL-induced upregulation of Nox4 is responsible for the increase in intracellular ROS formation and cytotoxicity observed in HMDMs exposed to OxLDL.

Nox4 Knockdown Protects HMDMs From the Cytotoxicity of OxLDL, Whereas Nox4 Overexpression Sensitizes HMDMs to OxLDL Toxicity

To confirm the role of Nox4 in OxLDL-induced cytotoxicity, we infected HMDMs with adenoviruses expressing either Nox4 siRNA or a scrambled control siRNA (scrRNAi).33 Nox4 siRNA completely suppressed OxLDL-induced Nox4 mRNA expression (not shown) and blocked Nox4 protein expression by 59% compared to scrRNAi (Figure 5A), but it did not affect Nox2 protein levels (Online Figure VII, A), confirming the specificity of the anti-Nox4 siRNA sequence. Knockdown of Nox4 reduced OxLDL-induced ROS formation by 39% and cytotoxicity by 42% (Figure 5B and 5C). In contrast, knockdown of Nox2 using antisense oligonucleotides resulted in a 33% reduction in total Nox2 protein levels (Online VII, B and C) but had no effect on OxLDL-induced cytotoxicity (Online Figure VII, D). Together, these results provide further evidence that Nox4 is the source of ROS that mediate OxLDL-induced macrophage death. Surprisingly, even though Nox4 siRNA routinely reduced baseline Nox4 mRNA levels by more than 50% in unstimulated HMDMs (not shown), Nox4 protein expression was not significantly decreased in these cells (Figure 5A), suggesting that the turnover of Nox4 in HMDMs is very slow. This finding also implies that the reduction in Nox4 levels by Nox4 siRNA that we observed in OxLDL-stimulated HMDMs is primarily attributable to the inhibition of de novo synthesized Nox4 expressed in response to OxLDL stimulation.
To increase constitutive Nox4 expression in macrophages, we generated a doxycycline-inducible adenoviral vector that contains both the Tet-on transcriptional activator and the Tet-responsive element (TRE) and carries the human Nox4 sequence. Induction of Nox4 expression with doxycycline (1 \( \mu \text{g/mL} \)) in HMDMs elevated total Nox4 protein levels by 41% (Figure 5D), which coincided with an increase of both OxLDL-induced intracellular ROS formation (+30%; Figure 5E) and cytotoxicity (+65%; Figure 5F). Nox4 overexpression did not affect Nox2 expression in HMDM (Online Figure VIII, A). In the absence of the adenoviral vector, doxycycline alone had no effect on Nox4 protein levels in HMDMs (Online Figure VIII, B) and did not affect ROS production or cell viability in HMDMs (not shown). These results confirm that Nox4 is the source of intracellular ROS production stimulated by OxLDL in HMDMs and that Nox4 induction mediates OxLDL-induced macrophage death. In the absence of OxLDL, induction of Nox4 expression did not significantly alter baseline ROS production in HMDMs or macrophage viability. This finding suggests that p22phox levels may be rate-limiting in resting HMDMs, but sufficient p22phox is synthesized in response to OxLDL stimulation to dimerize with the overexpressed transgenic Nox4.

**Discussion**

The goal of this study was to identify the source of the ROS that mediate OxLDL-induced cytotoxicity in macrophages. Here we demonstrated for the first time that human monocytes and macrophages express the NADPH oxidase Nox4 and that this enzyme is inducible by OxLDL. Our highly specific monoclonal antibody detected Nox4 not only in human blood monocytes and HMDMs, but also in mouse peritoneal macrophages. Nox4 was previously reported to be present in murine osteoclasts, suggesting that Nox4 may be expressed throughout the monocytic cell lineage. Compared to Nox2, the major source of ROS in phagocytic cells, Nox4 is expressed at lower levels in monocytes and HMDMs, which is consistent with the proposed role for Nox4 in cell signaling and may explain why previous studies failed to detect this enzyme in human monocytes. Confocal microscopy revealed that Nox4 was expressed throughout human and murine macrophages, again consistent with a potential role of this enzyme in redox signaling in human and mouse monocytic cells. We confirmed the localization of Nox4 in the ER previously reported by Chen et al. and also detected Nox4 in discrete sites within the nucleus, similar to findings in human airway smooth muscle cells reported by Sturrock et al. This latter finding is particularly intriguing as emerging evidence strongly suggests that processes located in the nucleus such as binding of transcription factors like NF-kB and AP-1 are redox regulated. We could not detect Nox4 protein in lysosomes (Online Figure VII), indicating that Nox4 expression is not ubiquitous within the cell and is instead limited to select intracellular membranes and organelles.

Most Nox family members, including Nox2, generate superoxide. However, using the redox sensitive dye DCFH-DA we were able to monitor changes in intracellular ROS that resulted from either increased or suppressed Nox4 expression. Thus, it appears that Nox4 produces \( \text{H}_2\text{O}_2 \) in macrophages. Although DCFH-DA is by no means specific, this dye does not react with superoxide and is selective for \( \text{H}_2\text{O}_2 \) though a catalyst is needed for \( \text{H}_2\text{O}_2 \) to oxidize DCFH-DA. Nevertheless, we cannot rule out that monocytic Nox4 is able to generate superoxide, which would dismutate to \( \text{H}_2\text{O}_2 \). In agreement with our findings, results from two recent studies suggest that Nox4 primarily produces \( \text{H}_2\text{O}_2 \), the primary ROS acting as the intracellular messenger in redox signaling. Together, its intracellular localization, inducibility and ability to generate \( \text{H}_2\text{O}_2 \) strongly suggest that as in nonphagocytic cells Nox4 may primarily perform signaling functions in macrophages.

We also identified Nox4 as the inducible source of ROS production required for OxLDL-induced macrophage death (Figure 6). This finding nicely illustrates the dichotomy of ROS: on the one hand, ROS are required for physiological redox signaling, yet they are harmful when generated in excess. Induction of Nox4 by OxLDL is mediated by the MEK/extracellular signal-regulated kinase (ERK) pathway, but the upstream components of this signaling mechanism in macrophage have not yet been clearly identified. Clavreul et al demonstrated that in endothelial cells, OxLDL activates the MEK/ERK pathway via the peroxynitrite-induced S-glutathionylation of p21ras. OxLDL promotes S-glutathionylation in HMDMs, but p21ras S-glutathionylation has not been reported in HMDMs. Although intriguing, this mechanism would place yet another ROS-generating system upstream of OxLDL-mediated Nox4 expression and Nox4-dependent ROS production.

Nox4 requires a second transmembrane protein, p22phox, to form a functional ROS-generating enzyme complex. We showed not only that Nox4 colocalizes with p22phox in HMDMs, but that Nox4 and p22phox showed similar induction profiles in response to OxLDL. This suggests that a majority of Nox4 in HMDMs is present as Nox4/p22phox.

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**Figure 6.** Hypothetical model for the role of Nox4 in OxLDL-mediated macrophage cytotoxicity. OxLDL induces Nox4 expression via the MEK/ERK pathway and is blocked by MEK inhibitors UO126 and PD98059. Knockdown of Nox4 (Nox4 RNAi) protects macrophages from increased ROS production and cell injury. The putative site of action for the peroxyl radical scavenger Trolox is indicated.

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**Overexpression of Nox4 (pAdNox4) does not affect basal ROS production or macrophage viability but sensitizes macrophages to OxLDL-induced ROS formation and cell death.**
dimers and most likely is active. If that is the case, and if the majority of Nox4 is indeed generating H$_2$O$_2$, how then could this system serve its signaling functions? One possibility is that the target(s) is recruited to the membrane where the Nox4/p22phox dimer is localized. Alternatively, the Nox4/p22phox system could be assembled and transported in vesicles or similar structures, and could then be recruited to signaling complexes such as lipid raft, caveolae or focal adhesion sites. 

OxLDL accumulates in atherosclerotic lesions and promotes macrophage death, thereby contributing to the development of the necrotic core. 

Although increased intracellular ROS production is essential for OxLDL-induced macrophage death, 

Nox2 deficiency in macrophages does not reduce atherosclerotic lesion development in mice. 

It appeared paradoxical that macrophage-derived ROS would not contribute to this process as the report from Kirk et al would suggest, especially in light of the large body of evidence linking oxidative stress to the development of atherosclerosis, as well as our recent finding that the cellular thioredox system of macrophages is a strong predictor of lesion development. With the identification of Nox4 as an alternative source of ROS and a mediator of macrophage death, our data offer a possible explanation for this paradox and suggest that the role of macrophage-derived ROS in atherogenesis should be revisited.

In summary, we identified Nox4 as a novel, inducible source of ROS in monocytes and macrophages, and we demonstrated that Nox4-derived ROS mediate OxLDL-induced macrophage death. Our results suggest that monocycte Nox4 may play an important role in macrophage function and the development and progression of atherosclerosis.

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Disclosures

None.

References

In addition to the classic phagocytic NADPH oxidase Nox2, we first to document Nox4 in human monocytes and macrophages implicating Nox4 in nuclear redox signaling. These data are the first to document Nox4 in human monocytes and macrophages and to identify a role for Nox4 in macrophage redox signaling and cell death.
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Online Figure I: Rabbit monoclonal antibodies directed against Nox4 (Nox4 mAb) detect endogenous Nox4 protein in both human and mouse tissues. The following samples were analyzed by Western blot: Lane 1: HEK293 cells; Lane 2: HEK293 cells transfected with pcDNA3-hNox4. Note that only overexpression of Nox4 resulted in the appearance of additional bands, possibly due to the posttranslational processing of Nox4 protein expressed in unphysiological levels. To demonstrate the specificity of the Nox4 mAb we also analyzed lysates of lung endothelial cells (EC) isolated from wildtype (Lane 3) and Nox4 knockout mice (Lane 4). The EC cell lysates were kindly provided by Dr. Ralph Brandes, Johann-Wolfgang Goethe Universität, Frankfurt, Germany. Lane 5: Human monocyte-derived macrophages (HMDM). Lane 6: Mouse peritoneal macrophages (MPM). * identifies the Nox4 band with a predicted MW of 66 kD.
Online Figure II: Nox4 is present in the nuclear fraction of human monocyte-derived macrophages. Nuclear fractions (Nuc) were prepared from HMDM as described under “Methods”. Whole cell lysates (WCL) and lysates of the nuclear fractions were separated by SDS-PAGE and probed for Nox4, nuclear markers histone 3 (H3) and lamin A, the cytosolic protein GAPDH, and the mitochondrial marker cytochrome c oxidase subunit IV (Cox IV).
Online Figure III: Nox4 colocalizes with CD68-stained macrophages in atherosclerotic lesions from LDL-receptor null mice. Aortic roots were isolated from four LDL-receptor-deficient mice fed a high fat diet (21% fat wt/wt; 0.15% cholesterol wt/wt; AIN-76A, BioServ) for 12 weeks. Frozen sections were prepared and subjected to multi-label fluorescence immunohistochemistry. Panel A: Representative cross-section of an aortic root showing an atherosclerotic lesion in one of the sinuses. Panels B-D show enlarged images of a selected area (see orange box in Panel A) on the adjacent section, which was stained with our monoclonal antibodies directed against Nox4 (red), a macrophage-specific rat antiserum directed against CD68 (green, Serotech) and DAPI (blue) to identify nuclei. Colocalization of Nox4 with CD68-positive areas (macrophages) are shown in yellow and indicated by arrows in the merged image (Panel B).
Online Figure IV: Panel A: OxLDL does not induce Nox2 expression. HMDM were stimulated with OxLDL for 6 h at the concentrations indicated. Nox2 protein levels were detected by Western blot analysis.

Panel B: Native LDL does not induce Nox4 expression. HMDM were incubated for 18 h with freshly isolated native LDL (100 µg/ml) OxLDL. Nox4 protein levels were detected by Western blot analysis.

Panels C and D: Lack of effect of Trolox on OxLDL-induced Nox4 expression. Trolox was present for 2 h prior to and during OxLDL stimulation and protected against OxLDL cytotoxicity (not shown) as reported previously (Asmis and Begley; Circ Res. 2003;92:e20-e29). Nox4 protein levels were detected by Western blot analysis. Results from 3 independent Western blot experiments are shown in panels B and C as mean ± standard deviation. * P <0.05 versus vehicle (PBS).
Online Figure V: Lack of effect of JAK2 inhibition on OxLDL-induced Nox4 expression and macrophage injury. HMDM were preincubated for 2 h with either vehicle (DMSO) or the JAK2 inhibitor AG490 (20 μM) and stimulated for 6 h (Nox4 expression) or 18 h (cytotoxicity) with OxLDL (75 μg/ml). Panel A: Nox4 protein levels were detected by Western blot analysis. Panel B: OxLDL cytotoxicity in HMDM was measured using the adenine release assay described under “Methods”.
Online Figure VI: Effect of MEK Inhibition on p22phox and Nox2 expression in HMDM. OxLDL induced the expression of p22phox but did not alter Nox2 protein levels. Expression of p22phox induced by OxLDL was blocked when macrophages were treated with the MEK inhibitor U0126.
Online VII: Specificity of knockdown approaches for Nox2 and Nox4. Knockdown of Nox4 was achieved as described in Figure 5. To knockdown Nox2, HMDM were pre-incubated overnight with oligonucleotides (50 nM) with either scramble sequence (5’ CATTGTGGAGTGACAGGAG 3’) or a Nox2 antisense sequence (5’ AACTGGGCTGTGAATGAGG 3’). HMDM were then stimulated for 18 h with 75 μg/ml OxLDL. Cell lysates were subjected to Western blot analysis and cytotoxicity assays were performed as described in “Methods”. Panel A: Knockdown of Nox4 did not affect Nox2 protein levels in HMDM. Panel B and C: Conversely, antisense oligonucleotides directed against Nox2 reduced Nox2 protein levels but did not affect basal Nox4 protein levels and did not inhibit Nox4 expression induced by OxLDL. Panel D Nox2 knockdown did not protect HMDM from OxLDL-induced cell injury.
Online Figure VIII: Panel A: Adenovirus-mediated Nox4 expression in human monocyte-derived macrophages. Adenovirus infection and addition of doxycycline (Dox)-induced Nox4 overexpression in HMDM carrying a Dox-inducible adenoviral vector had no effect on Nox2 protein expression levels. Panel B: Addition of doxycycline alone did not alter Nox4 expression in HMDM that were not infected with the inducible adenoviruses.
Online Figure IX Nox4 is not expressed in lysosomes of human monocyte-derived macrophages. **Panel A:** HMDM were fixed and stained with antibodies directed against Nox4 (red) and the lysosomal protein Lamp-1 (green) and confocal microscope images were taken. No colocalization (yellow) was observed (see Overlay). **Panel B** HMDM lysates were prepared and Nox4 protein was immunoprecipitated as described under “Methods”. Immunoprecipitates were subjected to Western blot analysis and probed for Nox4 and of Lamp-1 as described in “Methods”. Co-immunoprecipitation confirmed that Nox4 did not associate with Lamp-1.