An Oxidized Extracellular Oxidation-Reduction State Increases Nox1 Expression and Proliferation in Vascular Smooth Muscle Cells Via Epidermal Growth Factor Receptor Activation

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Objective—To examine the effect of an oxidized extracellular oxidation-reduction (redox) state (Eh) on the expression of NADPH oxidases in vascular cells.

Methods and Results—The generation of reactive oxygen species by NADPH oxidase (Nox)-based NADPH oxidases activates redox-dependent signaling pathways and contributes to the development of “oxidative stress” in vascular disease. An oxidized plasma redox state is associated with cardiovascular disease in humans; however, the cellular mechanisms by which the extracellular redox state may cause disease are not known. Aortic segments and cultured aortic smooth muscle cells were exposed to Eh between −150 mV (reduced) and 0 mV (oxidized) by altering the concentration of cysteine and its disulfide, cystine, the predominant redox couple in plasma. A more oxidized Eh increased the expression of Nox1 and resulted in Nox1-dependent proliferation of smooth muscle cells. Oxidized Eh rapidly induced epidermal growth factor receptor phosphorylation via shedding of epidermal growth factor–like ligands from the plasma membrane and caused extracellular signal–regulated kinase 1/2–dependent phosphorylation of the transcription factors activating transcription factor-1 and cAMP-response element–binding protein. Inhibition of epidermal growth factor receptor or extracellular signal–regulated kinase 1/2 activation, or addition of small interference RNA to activating transcription factor-1, prevented the increase in Nox1 expression.

Conclusion—Our results identify a novel mechanism by which extracellular oxidative stress increases expression and activity of Nox1 NADPH oxidase and contributes to vascular disease. (Arterioscler Thromb Vasc Biol. 2010;30:2234-2241.)

Key Words: oxidative stress • atherosclerosis • NADPH oxidase • epidermal growth factor receptor

Reactive oxygen species (ROS) contribute to the pathogenesis of cardiovascular diseases, including hypertension, atherosclerosis, cardiac hypertrophy, heart failure, and restenosis.1–3 An increase in cellular ROS contributes to the pathogenesis of vascular disease by altering endothelial cell function, enhancing vascular smooth muscle cell (SMC) growth and proliferation, stimulating the expression of proinflammatory genes, and modulating reconstruction of the extracellular matrix.1–4 Although an excessive amount of ROS is considered harmful and contributes to the pathogenesis of disease, ROS normally participate in the regulation of important cellular processes, including cell signaling, gene expression, cellular death and senescence, regulation of growth, oxygen sensing, activation of matrix metalloproteinases (MMPs), and angiogenesis.5–7

Although several cellular sources of ROS have been identified, the primary source of ROS in vascular cells is NADPH oxidases.3,8 Of the Nox isoforms, Nox1, Nox2, Nox4, and Nox5 are expressed in vascular cells, with expression patterns showing cell specificity. For example, only Nox1 and Nox4 are expressed in rodent aortic SMCs.9 The activity of the vascular NADPH oxidases is regulated by cytokines, hormones, and mechanical forces known to be involved in the pathogenesis of vascular disease.10,11 NADPH oxidase expression is associated with the progression of atherosclerosis in humans12,13 and with plaque instability in human coronary arteries.14 Essential cellular processes, such as proliferation, differentiation, and apoptosis, are regulated by reversible oxidation-reduction (redox) reactions of thiol/disulfide couples.15–17 The most abundant intracellular low-molecular-weight thiol/disulfide couple is glutathione (GSH)/GSSG disulfide (GSSG), which regulates a variety of cellular processes. After adjusting for traditional risk factors, the
redox state (Eh) of GSH/GSSG was an independent predictor for carotid intima-media thickness in healthy patients. In contrast, the predominant extracellular low-molecular-weight thiol/disulfide couple is cysteine and cystine (Cys/CySS). In humans, the redox state of plasma Cys/CySS differs considerably between individuals, with an oxidized Eh Cys/CySS in patients with increasing age, cigarette smoking, and type 2 diabetes mellitus, all of which are risk factors for the development of cardiovascular disease. In addition, levels of oxidized Cys metabolites correlated with endothelial dysfunction in a healthy human population. Recently, oxidized extracellular Eh Cys/CySS determined proinflammatory cytokine levels in human plasma. However, the effect of the extracellular redox state on the expression or activity of vascular NADPH oxidases is not known.

The goal of this study was to examine the effect of extracellular oxidative stress (oxidized Eh) on NADPH oxidases in SMCs. Our findings identify a signaling pathway by which oxidation of the extracellular environment activates epidermal growth factor receptor (EGFR) through metalloproteinase-mediated shedding of EGF-like ligands, leading to subsequent activation of the transcription factor activating transcription factor-1 (ATF-1) and subsequent expression of Nox1. These data identify a novel mechanism by which extracellular oxidative stress activates redox-sensitive pathways that increase Nox1-mediated NADPH oxidase expression and SMC proliferation.

Methods

The supplemental data (available online at http://atvb.ahajournals.org) provide a complete description of materials and methods used for these studies. Medial SMCs were cultured from thoracic aorta of C57BL/6 mice. The range of extracellular thiol/disulfide redox states (from −150 to 0 mV) was generated by varying concentrations of Cys and CySS added to cyst(e)ine-free DMEM, as previously described, while maintaining a constant concentration of total Cys moieties of 200 μmol/L. Recombinant adenoviruses encoding small interfering Nox1 were cotransfected into SMCs with plasmid containing SV40 promoter (pSV40)-basic (empty vector) or Nox1 promoter–luciferase plasmid. Data were reported as a ratio between the firefly and Renilla luciferase. Statistical comparisons were performed by Student 2-tailed t tests or ANOVA, with a Dunnett or Tukey multiple comparison posttest as appropriate. P<0.05 was considered significant.

Results

Oxidized Extracellular Eh Increases Nox1 Expression and SMC Growth

Exposure of isolated aortas to an oxidized extracellular redox environment increased cellular outgrowth and expression of the NADPH oxidase catalytic subunit Nox1 (Figure 1A). Because SMCs are the predominant source of Nox1 in the blood vessel, subsequent experiments focused on characterizing the response of cultured SMCs to changes in extracellular redox. Similar to observations in aorta segments, SMC expression of Nox1 increased in response to an increasingly oxidized extracellular redox state. At the oxidized Eh of 0 mV, Nox1 mRNA expression increased >2-fold after 24 hours (supplemental Figure IA) and >4-fold after 48 hours (Figure 1B) compared with the Eh of −150 mV. An increase in Nox1 protein expression was confirmed by Western blotting (Figure 1C and supplemental Figure IC and D). In contrast to Nox1, SMC expression of Nox4 was not significantly affected by changes in the extracellular redox state (Figure 1B and supplemental Figure IB). Oxidized extracellular Eh failed to increase Nox1 levels in human embryonic kidney 293 or human gut epithelial carcinoma cells, identifying cell specificity to this response (supplemental Figure IE). Next, we determined whether the oxidized Eh–mediated increase in SMC Nox1 resulted in an increase in cellular generation of ROS. Compared with the more reduced extracellular Eh, oxidized Eh increased superoxide levels in SMCs; this effect was prevented by the selective inhibition of Nox1 expression (Figure 1D and E). Because Nox1 has been implicated in regulating cell growth, we then examined whether an oxidized Eh induced SMC proliferation. Compared with more reduced conditions (−150 mV), an oxidized Eh (0 mV) for 48 hours resulted in >1.5-fold increase in thymidine incorporation, which was dependent on the expression of Nox1 (Figure 1F). These data indicate that exposure of vascular SMCs to an oxidized extracellular environment increases expression and activity of Nox1, resulting in increased levels of ROS and cell proliferation.

Oxidized Extracellular Eh Activates EGFR Via MMPs/Disintegrin and Metalloproteinase Family

The EGFR is a primary coordinator of signal transduction from the extracellular environment. Therefore, we examined activation of the EGFR as a potential mediator of oxidized extracellular Eh–dependent increase in Nox1 gene expression. Oxidized extracellular Eh rapidly induced transient EGFR phosphorylation (Figure 2A and supplemental Figure IIA), confirmed by the specific inhibitor of EGFR phosphorylation AG1478 (supplemental Figure IIB). Many ligands of the EGFR are synthesized as transmembrane precursors that must be cleaved by metalloproteinases to release mature ligands. Two closely related metalloproteinase families, MMPs and a disintegrin and metalloproteinase family (ADAMs), contain critical redox-sensitive Cys residues. We tested whether oxidized extracellular Eh could activate metalloproteinases, thereby releasing surface-bound EGFR ligands. Rapid phosphorylation of the EGFR in response to oxidized extracellular Eh was attenuated by the general metalloproteinase inhibitor GM6001 (Figure 2B), confirming involvement of surface metalloproteinases in EGFR activation to oxidative stress, consistent with previous reports. Next, we used the fluorogenic peptide substrate III converting enzyme (or ADAM17) and related enzymes (eg, ADAM9 and ADAM10) to investigate the role of specific metalloproteinases in the activation of EGFR. Studies of SMCs exposed to oxidized extracellular Eh in the presence of a fluorogenic substrate selective for tumor necrosis factor α converting enzyme (or ADAM17) and related enzymes (eg, ADAM9 and ADAM10) indicated activation of ADAMs within minutes, which per-
sisted for several hours (Figure 2C). Similarly, studies using a fluorogenic substrate selective for MMPs suggested rapid and sustained activation of these metalloproteinases to oxidize extracellular Eh (Figure 2C). Additional evidence for MMP activation is provided by the finding that oxidized extracellular Eh increased proteolytic activity of MMP-2 and MMP-9 and their proforms (Figure 2D). These data indicate that exposure of SMCs to an oxidized extracellular environ-

**Figure 1.** Oxidized extracellular Eh increases Nox1 expression and growth of vascular cells. Isolated aortas and SMCs were exposed to varying concentrations of Cys and CySS to obtain a range of Eh from $-150$ to $0$ mV. A, Representative images show aortic outgrowth on Matrigel. Mean outgrowth area was calculated from 8 aortic rings in each group, obtained from 2 animals. Nox1 mRNA expression was measured by quantitative RT-PCR (qRT-PCR) (n=4). B, Nox1 and Nox4 mRNA expression in SMCs was measured by qRT-PCR (n=4). C, Nox1 protein expression in SMCs was measured by Western blotting. Results are normalized to GAPDH expression (n=6). D, Superoxide levels in SMCs were assessed by dihydroethidium (DHE) fluorescence. E, Mean DHE fluorescence was obtained from 14 to 16 fields per group in 2 independent experiments. F, SMC proliferation was measured by $[^3]H$-thymidine incorporation (n=4). Data are presented as mean±SE. *P<0.05 vs $-150$ mV and $P<0.05$ vs $0$ mV Ad-empty. Ad-siNox indicates recombinant adenoviruses encoding small interference Nox1. Ad-empty indicates an empty expression cassette adenovirus.

**Figure 2.** EGFR is phosphorylated by oxidized extracellular Eh via activation of metalloproteinases. A, SMCs were treated with Eh=$0$ mV and p-EGFR expression analyzed by Western blotting. Results are normalized to total EGFR expression (n=4). B, SMCs were incubated with 20-$\mu$mol/L GM6001 and then treated with Eh=$0$ mV for 1 minute. Phosphorylated EGFR was analyzed by Western blotting. Results are normalized to total EGFR expression (n=4). Blots shown under A and B were run on the same gel but cut for better presentation. C, Fluorogenic peptide substrates were added to Eh=$0$ mV media, then collected at specific points for measurement of metalloproteinase activity. An appropriate blank (without fluorogenic substrate) was subtracted from each measurement (n=5). D, Gelatin zymography of MMP activity after control (c) and Eh=$0$ mV (f). A representative gel of 3 independent experiments is shown. Summary data are presented as mean±SE. *P<0.05 vs untreated (0 minutes) and $\Delta P<0.05$ vs $0$ mV vehicle. p- indicates phosphorylated form of the protein; pro-MMP, latent form of the MMP.
Oxidized extracellular Eh activates both the MMP and ADAM families of metalloproteinases, resulting in phosphorylation of the EGFR via shedding of EGF-like ligands from the cell surface. The addition of EGF ligands to SMCs increases intracellular ROS. We examined the effect of short-term changes in extracellular Eh on intracellular ROS levels. Forty-five minutes after exposure to 0 mV extracellular Cys/CySS (versus −150 mV), cellular 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFH) fluorescence was increased 43±9% (n = 5, P < 0.05).

Figure 3. Oxidized extracellular Eh increases Nox1 expression via the metalloproteinase-EGFR pathway. A and B, SMCs were pretreated with 10-μmol/L AG1478 (A) or 20-μmol/L GM6001 (B) and then exposed to Eh as indicated for 24 hours. Nox1 mRNA expression was measured by quantitative RT-PCR (n = 4). C and D, SMCs were transfected with Nox1 promoter luciferase or control plasmid, then pretreated with 10-μmol/L AG1478 (C) or 20-μmol/L GM6001 (D) and exposed to Eh as indicated for 8 hours. Firefly luciferase activity was normalized to Renilla luciferase (n = 4). Data are presented as mean±SEM. *P < 0.05 vs control and ΔP < 0.05 vs 0-mV vehicle.

Oxidized Eh Increases Nox1 Expression Via EGFR

Next, we determined whether phosphorylation of the EGFR was necessary for increasing expression of Nox1 in response to oxidized extracellular Eh. Inhibition of EGFR phosphorylation by the specific inhibitor AG1478 prevented the increase in Nox1 expression (Figure 3A). Moreover, consistent with our observation that oxidized extracellular Eh activates EGFR by metalloproteinase-mediated shedding of EGF-like ligands, the increase in Nox1 expression was abolished by GM6001 (Figure 3B). A role for EGFR activation in Nox1 expression is further supported by the observation that AG1478 and GM6001 prevented an increase in Nox1 promoter–luciferase activity (Figure 3C and D).

Oxidized Extracellular Eh Activates ERK1/2 and Akt

Members of the MAPK family and phosphatidylinositol 3-kinase/Akt are downstream targets of EGFR activation. Therefore, we investigated the involvement of these signaling pathways in mediating an increase in Nox1 expression. Oxidized extracellular Eh activates ERK1/2 and Akt in a time-dependent manner, with maximal phosphorylation within the first several minutes (supplemental Figure IIIA and B), which was abolished by pretreatment with GM6001 (Figure 4A and D) or with AG1478 (Figure 4B and E). These data demonstrate that oxidized extracellular Eh activates ERK1/2 and Akt through shedding of EGF-like factors and subsequent activation of EGFR. Inhibiting ERK1/2 activation by the selective MAPK/ERK kinase inhibitor U0126 prevented the increase in Nox1 promoter–luciferase activity to oxidized extracellular Eh (Figure 4C), whereas inhibition of the phosphatidylinositol 3-kinase/Akt pathway by wortmannin had no effect (Figure 4F). A role for ERK1/2 in expression of Nox1 was further supported by the observation that expression of a constitutively active form of MAPK/ERK kinase in SMCs markedly increased expression and promoter-luciferase activity of Nox1 (supplemental Figure IV).

Oxidized extracellular Eh had no effect on p38 MAPK (supplemental Figure IIII) but caused a modest activation of janus kinase (JNK) (supplemental Figure IIID). However, inhibition of the JNK MAPK pathway with SP600125, a potent JNK inhibitor, had no effect on Nox1 expression and promoter-luciferase activity (data not shown). Together, these results suggest that in response to changes in extracellular redox state, signal transduction from the cell surface to the transcription of Nox1 primarily involves activation of the EGFR-ERK1/2 pathway.

Oxidized Extracellular Eh Activates Transcription Factors ATF-1 and CREB

It was previously shown that prostaglandin F2α and platelet-derived growth factor (PDGF) increase Nox1 expression via ATF-1. Therefore, we examined the role of ATF-1 and its related transcription factor CREB in oxidized Eh-induced Nox1 expression. After extraction of DNA-bound proteins from the nucleus, both CREB and ATF-1 were observed to be phosphorylated by oxidized extracellular Eh in a time-dependent manner (Figure 5A). After construction and testing of small interference RNAs to the transcription factors CREB and ATF-1 (supplemental Figure V), we found that small interference RNA to CREB had no effect (Figure 5C). Then, we determined if oxidized extracellular Eh activated ATF-1 via the EGFR-ERK1/2 signaling pathway. Both
GM6001 and AG1478 prevented activation of ATF-1 (Figure 6A and supplemental Figure VI); the same inhibitors failed to significantly prevent activation of CREB (Figure 6D and supplemental Figure VI). Similarly, phosphorylation of ATF-1, but not CREB, was inhibited by U0126 (Figure 6B and E and supplemental Figure VI). Consistent with our findings that phosphatidylinositol 3-kinase/Akt was not involved in activation of Nox1 expression to oxidized extracellular Eh (Figure 4F), wortmannin failed to prevent the increase in ATF-1 or CREB phosphorylation (Figure 6C and F and supplemental Figure VI). Taken together, these results identify a signaling pathway by which oxidation of the extracellular environment activates EGFR through metalloproteinase-mediated shedding of EGF-like ligands, leading to ERK1/2-dependent activation of the transcription factor ATF-1 and subsequent expression of Nox1 (supplemental Figure VII).

Discussion
In humans, the redox state (Eh) of plasma Cys/CySS differs considerably between individuals, ranging from approximately −130 to −20 mV, with a Cys/CySS redox potential in young healthy individuals estimated at −80 mV. Increased age,19 cigarette smoking,20 and diabetes.17 In this study, we describe a signaling pathway in vascular SMCs by which oxidation of extracellular Cys/CySS activates EGFR through metalloproteinase-mediated shedding of EGF-like ligands, leading to ERK1/2-dependent activation of the transcription factor ATF-1 and subsequent expression of Nox1 NADPH oxidase. These findings establish a novel mechanism by which extracellular oxidative stress induces SMC activation and proliferation in vascular disease (supplemental Figure VII).

The Cys/CySS redox couple represents a nonradical redox system involved in the regulation of cell function. In contrast to the high flux electron transfer pathways of NADH and NADPH, the thiols and disulfides are low flux. Plasma disulfide CySS predicts adverse cardiovascular outcomes. Among markers of oxidative stress, plasma CySS and a more oxidized GSH/GSSG ratio correlated with intima-media thickness.18 Plasma CySS and mixed disulfide levels were inversely correlated with endothelium-dependent vasodilation in a healthy human population.21 In addition, oxidized extracellular Eh Cys/CySS has been associated with nuclear factor κB activation, monocyte binding to endothelial cells,39 and production of inflammatory cytokines in human plasma.22 Our study provides a potential cellular mechanism for these findings by showing that an oxidized extracellular
redox environment increases Nox1 expression, intracellular ROS, and SMC growth.

Increased expression of NADPH oxidase is associated with development of atherosclerosis and plaque instability in human arteries. Several studies have implied Nox1 in the pathogenesis of vascular disease. Nox1 expression and vascular ROS are increased early after balloon injury of rat carotid artery and associated with neointimal hyperplasia. Nox1 has an important role in the migration and proliferation of SMCs and deficiency of Nox1 reduced wire-induced neointima formation by >50%. In addition, overexpression of Nox1 in SMCs augments angiotensin II–induced hypertension and hypertrophy, and ROS derived from Nox1 reduces bioavailability of NO. Thus, the ability of oxidized extracellular Eh Cys/CySS to increase Nox1 expression in SMCs has important clinical relevance in the pathophysiological features of vascular disease.

The effects of an oxidized extracellular environment on NADPH oxidase appear to be cell type and subunit specific. We found that oxidized extracellular Eh Cys/CySS increases expression of Nox1 in SMCs while having no effect on the expression of the Nox4 isoform. In addition, an oxidized extracellular Eh failed to alter expression of Nox1 in human embryonic kidney 293 and human gut epithelial carcinoma cells, 2 distinctly different cell types, the first having low levels of Nox1 and the second having relatively high levels of Nox1. Although an oxidized Eh Cys/CySS increases proliferation of lung fibroblasts and SMCs (present study), human colon carcinoma, human retinal pigment, and NIH 3T3 cells proliferate more rapidly in conditions of reduced extracellular Eh Cys/CySS. The explanation for these discordant growth responses among different cell types to changes in extracellular redox potential is not known.

Several cell surface proteins are modified in response to changes in extracellular Cys/CySS redox state by the oxidation of membrane thiols. The activation of intracellular kinases and early mitogenic signaling events involve thiol-mediated activation of cell surface proteins. Our data confirm the activation of metalloproteinases by an oxidized extracellular Eh Cys/CySS with subsequent shedding of EGF-like ligands and phosphorylation of the EGFR. Interestingly, changes in extracellular redox potential are sufficient to activate this mitogenic pathway in the absence of growth factors. Changes in extracellular Eh Cys/CySS are not accompanied by changes in intracellular GSH/GSSG redox state and the extracellular Cys/CySS redox state is not regulated by the cellular GSH pool.

Figure 5. Oxidized extracellular Eh activates transcription factors CREB and ATF-1; however, only ATF-1 contributes to the increase in Nox1 expression. A, SMCs were treated with Eh_0 mV, and the nuclear fraction was subjected to Western blotting for p-CREB and p-ATF-1. A representative blot of 3 independent experiments is shown. B and C, SMCs were transfected with 50-nmol/L small interference RNA (siRNA) to ATF-1 (B) or CREB (C) and exposed to Eh_0 mV for 24 hours. Nox1 mRNA expression was measured by quantitative RT-PCR. Scrambled siRNA served as a control. Data are presented as mean±SE (n=5). *P<0.05 vs control and ΔP<0.05 vs 0 mV control siRNA. p- indicates phosphorylated form of the protein.

Figure 6. ATF-1, but not CREB, is activated via the EGFR-ERK1/2 pathway in response to oxidized extracellular Eh_0 mV. A, SMCs were pretreated with 10-μmol/L AG1478 (A and D) or 1-μmol/L wortmannin (C and F) and stimulated with Eh_0 mV. Summary data of p-ATF-1 and p-CREB expression analyzed by Western blotting are shown. Results are normalized to GAPDH expression and presented as mean±SE (n=3 to 5). *P<0.05 vs control and ΔP<0.05 vs 0 mV vehicle.
Previous studies have shown that oxidized extracellular E$_\text{r}$ Cys/CySS increases intracellular ROS levels in endothelial cells,$^{39}$ monocytes,$^{22}$ and mouse embryonic fibroblasts.$^{49}$ In a recent study,$^{49}$ extracellular oxidizing conditions induced mitochondria-derived ROS. We found that Nox1 was responsible for the observed increase in intracellular ROS after exposure of SMCs to oxidized extracellular E$_\text{r}$ Cys/CySS. These observations are not incompatible and raise the possibility of ROS-induced ROS.

Oxidized extracellular Cys/CySS induced strong activation of the ATF-CREB transcription factors; however, only ATF-1 (not CREB) was necessary for the observed increase in Nox1 expression. This finding is consistent with previous reports,$^{57,55}$ showing that upregulation of Nox1 expression was mediated by ATF-1. Transactivation of the EGFR and subsequent activation of ERK1/2 are common events in ATF-1 induction of Nox1 expression by multiple stimuli, including prostaglandin F$_{2\alpha}$, PDGF,$^{57}$ and an oxidized extracellular environment (present study). Despite the presence of both CREB-1 and ATF-1 in vascular SMCs, only ATF-1 was found in the CRE-DNA complexes formed in response to PDGF and thrombin.$^{58}$ Similarly, although PDGF and thrombin induced phosphorylation of both CREB and ATF-1, CREB phosphorylation did not contribute to cell growth.$^{58}$ Instead, CREB was involved in SMC differentiation and is a negative regulator of SMC growth.$^{59}$ CRE-regulated gene transcription is a complex process involving CRE/ATF-1 binding to the CRE, CREB/ATF-1 phosphorylation, and interaction with coactivator proteins.$^{59}$ The regulation of coactivators of CREB/ATF-1 is not well understood and may explain the distinct transcriptional involvement of CREB and ATF-1 in SMCs.

In summary, our findings identify a signaling pathway in vascular SMCs by which oxidation of the extracellular environment activates EGFR through metalloproteinase-mediated shedding of EGF-like ligands, leading to ERK1/2-dependent activation of the transcription factor ATF-1 and subsequent expression of Nox1. These data identify a novel mechanism by which extracellular oxidative stress, through oxidation of the Cys/CySS couple, can activate redox-sensitive pathways that stimulate NADPH oxidase expression and vascular SMC proliferation.

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Disclosures
None.

References


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Supplement Material

Chemicals and Antibodies. Fetal bovine serum (FBS), Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin mixture, L-glutamine, trypsin/EDTA and HEPES buffer solution were purchased from Gibco BRL (Grand Island, NY). [Methyl-³H] thymidine (1.0 mCi/ml) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Complete protease inhibitor cocktail was obtained from Roche Applied Science (Indianapolis, IN). BD Matrigel Matrix was purchased from BD Biosciences (Bedford, MA). Anti-phospho-EGFR antibody (pY¹⁰⁶⁸) was purchased from BioSource (Camarillo, CA). Antibodies to EGFR, phospho-Akt (pS⁴⁷³), phospho-CREB (pS¹³³, reactive with phosphorylated forms of CREB and ATF-1), and MEK1/2 were from Cell Signaling Technology (Danvers, MA). Anti-GAPDH antibody was purchased from Chemicon International (Temecula, CA). Anti-Nox1, anti-phospho-ERK1/2 (pT²⁰²/pY²⁰⁴), anti-ERK1/2, anti-phospho-p38 (pY¹⁸²), and anti-phospho-JNK (pT¹⁸³/pY¹⁸⁵) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary horseradish peroxidase-conjugated goat anti-mouse, rabbit anti-goat, and goat anti-rabbit antibodies were from Bio-Rad Laboratories (Hercules, CA). Bio-Rad D₃ Protein Assay kit was used for protein measurement. Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Except for wortmannin (Sigma-Aldrich; St. Louis, MO), all other pharmacologic inhibitors were obtained from Calbiochem (San Diego, CA). TRIzol Reagent and SuperScript II Reverse Transcriptase kit were from Invitrogen (Carlsbad, CA). DNA-free kit was purchased from Ambion (Austin, TX), whereas PCR Nucleotide Mix was obtained from Roche Applied Science (Indianapolis, IN). Dihydroethidium (DHE) and 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein
diacetate, acetyl ester (CM-H$_2$DCFDA) were from Molecular Probes (Eugene, OR), whereas Vectashield H-1000 mounting medium was from Vector Laboratories (Burlingame, CA). Fluorogenic peptide substrates were purchased from R&D Systems (Minneapolis, MN), whereas Centricon Ultracel YM-10 filter tubes were obtained from Millipore Corporation (Billerica, MA). The Dual Luciferase Assay Kit was from Promega (Madison, WI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Animals and Cells.** Mice were housed in an AALAC accredited University Animal Care Facility and all procedures were approved by the Institutional Animal Care and Use Committee at University of Iowa and complied with the standards stated in the Guide for the Care and Use of Laboratory Animals. Thoracic aortae were removed from 12 week old C57BL/6 mice and transferred to cold cyst(e)ine-free DMEM containing 0.2% (v/v) FBS, 4 mM glutamine, 10 U/ml penicillin, and 10 $\mu$g/ml streptomycin. The loosely adhering fibroadipose tissue was removed and aorta cut into 1 mm long rings. Vessel segments were placed in redox media (-150 or 0 mV; see below) at 37°C and the media replaced after 12 hours. After 24 hours of incubation with the redox media, some aortic segments were prepared for isolation of total mRNA for real time PCR while others for aortic outgrowth experiments (see below).

Medial smooth muscle cells (SMCs) were cultured from thoracic aorta of C57BL/6 mice by enzymatic digestion, as described previously ($^1$). Cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 10 U/ml penicillin, 10 $\mu$g/ml streptomycin, 4 mM glutamine, and 20 mM HEPES, at 37°C and 5% CO$_2$. SMCs were confirmed positive by $\alpha$-actin
staining at second passage. All experiments were conducted using SMCs between passages 6 and 11 and 60-90% confluence.

**Preparation of Redox Media.** The range of extracellular thiol/disulfide redox states (from -150 to 0 mV) was generated by varying concentrations of cysteine (Cys) and cystine (CySS) added to cyst(e)ine-free DMEM (Sigma-Aldrich; St. Louis, MO) containing 0.2% (v/v) FBS, 4 mM glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin, as described previously. Essentially, concentrations of Cys and CySS were calculated using the Nernst equation:

\[ E_h = E_o + \frac{RT}{2F} \ln \left( \frac{[\text{CySS}]}{[\text{Cys}]^2} \right) \]

where \( E_h \) represents the electromotive force relative to a standard hydrogen electrode, \( R \) is the gas constant, \( T \) is the absolute temperature, \( F \) is Faraday’s constant, and \( E_o \) is the standard electrode potential (−250 mV at pH 7.4) for the Cys/CySS redox couple. The total concentration in cysteine moieties was kept constant, i.e. \([\text{Cys}] + 2 \times [\text{CySS}] = 200 \mu M\). Medium pH was adjusted to 7.4 following Cys and CySS addition, and medium was filter-sterilized prior to use. The redox medium was prepared fresh every day and changed every 10-12 h after addition to cells or aortic segments.

**Adenoviral-mediated Gene Delivery.** Recombinant adenoviruses encoding siNox1 (Ad-siNox1, a gift from Dr. Robin Davisson, Cornell University) and constitutively active form of the mitogen-activated protein kinase kinases (Ad-caMEK, a gift from Dr. Noriaki Mitsuda, Ehime University, Japan) were used. Ad-empty or Ad-siGFP were used as controls. Adenovirus was mixed with the cationic polymer poly-L-lysine (250 molecules/virus particle) and added to SMCs (approximately 70% confluence) in serum free-DMEM. After 6 h, media were replaced
with DMEM containing 10% FBS, and after an additional 24-48 h exposed to varying $E_h$
Cys/CySS redox conditions per experimental protocol.

**Real-time PCR Analysis.** Detection of mRNA for Nox1 and Nox4 was performed using
quantitative real-time PCR (qRT-PCR). Smooth muscle cells were plated on 100-mm dishes and
cultured for 24 h in normal growth medium (at approximately 70% confluence), then serum-
deprived (0.2% FBS) for another 24 h before culture under varying $E_h$ Cys/CySS redox
conditions. Three mm thick aortic segments were incubated in different $E_h$ Cys/CySS redox
conditions for 24 h prior to RNA isolation. Total RNA from cells and tissues was isolated with
TRIzol reagent (Invitrogen, Carlsbad, CA) and chloroform followed by isopropanol
precipitation. Contaminating genomic DNA was removed with DNA-free reagent (Ambion,
Austin, TX). The reverse transcription reaction of the extracted RNA was performed by using
the SuperScript II Reverse Transcriptase kit from Invitrogen. Five microliters of cDNA from
first-strand reaction were used for qRT-PCR reaction, containing Power SYBR Green PCR
Master Mix (Applied Biosystems, Foster City, CA) and 0.1 µM forward and reverse primers in a
total volume of 25 µl. ROX was used as an internal reference dye. Primers were designed using
Primer Express Software and synthesized by Integrated DNA Technologies (Coralville, IA). The
following primers were used: Nox1 forward primer (TGGCATCCCTTCACTCTGACT), reverse
primer (AGTCCCCTGCTGCTCGAATA); Nox4 forward primer
(GTTGGGCCTAGGATTGTGTTTAA), reverse primer (AAAGGATGAGGCTGCAGTTGA).
Samples were run on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using
the following cycle program: 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40
cycles at temperatures of 95°C for 15 s and 60°C for 1 min. Results were quantitated using the
comparative cycle threshold (ΔΔCt) method. Ribosomal 18S mRNA was measured in the same samples (forward, CGCAGCTAGGAATAATGGAATAGG; reverse CATGGCCTCAGTTCCGAAA) and used to correct for variations in RNA content among samples.

**Measurement of Intracellular ROS.** Intracellular ROS levels were detected in SMCs with dihydroethidium (DHE) or 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA). In experiments studying the effects of prolonged exposure to different extracellular redox conditions on ROS production, SMCs grown on chamber slides were infected with adenoviruses as described above, then serum-deprived (0.2% FBS) for 24 h before stimulation with different E$_h$ Cys/CySS redox conditions for 48 h. On the day of study, cells were rinsed with PBS, treated with DHE (10 μM) for 1 h at 37°C, then rinsed with PBS before mounting the slides in Vectashield H-1000 mounting medium (Vector Laboratories; Burlingame, CA). DHE fluorescence intensity was visualized with Zeiss LSM 510 META laser confocal microscope as described. In each experiment, 7 to 8 randomly picked fields per sample (with 29-54 cells in each field) were visualized and captured. The resulting fluorescence was quantitated using NIH ImageJ software and expressed as relative fluorescence intensity per cell. ROS production after an acute exposure to different extracellular redox conditions was measured by CM-H$_2$DCFDA fluorescence. Medial SMCs were cultured on six-well plates for 24 h in normal growth medium at approximately 80% confluence, then serum-deprived (0.2% FBS) for another 24 h before loading with 20 μM CM-H$_2$DCFDA for 45 min. Cells were stimulated with different E$_h$ Cys/CySS redox conditions for 45 min, then rinsed with PBS, collected by trypsinization, centrifuged at 400 g for 5 min at 4°C, resuspended in ice-cold PBS, filtered, and
kept on ice in the dark for immediate analysis. Flow cytometric data were collected on a Becton Dickinson FACScan (BD Biosciences, San Jose, CA) flow cytometer and analyzed with the FlowJo software package (Tree Star, Inc., Ashland, OR).

[methyl-³H]-Thymidine Incorporation Assay. SMCs were incubated in serum-deprived medium (0.2% FBS) for 24 h before culture under different \( E_h \) Cys/CySS redox conditions for 48 h, with 0.05 µCi/ml of [methyl-³H]-thymidine added for the final 4 h. Afterward, the medium was removed, and the cells were washed with PBS, incubated with ice-cold trichloroacetic acid, washed again with PBS, and finally solubilized in NaOH. An aliquot of neutralized cell extract was counted in a Beckman Coulter LS6500 liquid scintillation counter.

Aortic Outgrowth Assay. After 24 hours in -150Mv or 0 mV \( E_h \) Cys/CySS redox conditions, aortic sections were embedded in 48-well plates coated with Matrigel Matrix (BD Biosciences) and incubated at 37°C in normal growth medium for 72 h. Images were captured by light microscopy at 4x magnification and the outgrowth area was analyzed using NIH ImageJ software.

Western Blot Analysis. DNA binding proteins⁸ or whole cell lysates³ were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were first incubated overnight at 4°C with a specific primary antibody followed by 1 hour incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Specific immunoreactive signals were detected using the enhanced chemiluminescence kit (ECL). Images were captured digitally and quantitated by densitometric analysis using NIH ImageJ software.
software. To obtain loading controls, the same membranes were stripped and then incubated overnight at 4°C with a primary antibody. Finally, the membranes were incubated with a HRP-conjugated secondary antibody for 1 hour and immune complexes were visualized using the Amersham ECL system. Results were expressed as the ratio of phosphorylated form of the protein to the appropriate loading control.

**Gene Silencing of CREB and ATF-1.** The 21-nt anti-CREB and anti-ATF-1 double-stranded small interference (si)RNAs were designed against nucleotides 663-683 of the mouse CREB mRNA sequence, transcript variant A (Accession number NM_133828) and against nucleotides 314-334 of the mouse ATF-1 mRNA sequence (Accession number NM_007497), respectively. Sense and antisense oligonucleotide sequences and their scrambled controls were designed using BLOCK-iT RNAi Designer and synthesized by the same company (Invitrogen). A complete list of siRNAs and their sequences is given in Table 1. Medial smooth muscle cells were plated on 100-mm dishes and cultured for 24 h in normal growth medium (at approximately 60% confluence). siRNAs were introduced into SMCs in a final concentration of 50 nM using Lipofectamine RNAiMAX reagent and OPTI-MEM I reduced serum medium, according to the manufacturer’s recommendations. After 5 h of incubation, transfection mixture was replaced with a normal growth medium for 24 h, cells were then serum-deprived (0.2% FBS) for another 24 h and used either for Western blotting or RT-PCR analysis.

**Table 1.** Sense and antisense siRNA sequences.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB siRNA - sense</td>
<td>5’-GCCCGAGCAACCAAGUUGUUTT-3’</td>
</tr>
<tr>
<td>CREB siRNA - antisense</td>
<td>5’-AAACAACUUGGUUGCUUGGGCTT-3’</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Scrambled CREB siRNA - sense</td>
<td>5’-GCCAACGAACCUUGACGUUTT-3’</td>
</tr>
<tr>
<td>Scrambled CREB siRNA - antisense</td>
<td>5’-AACGUCAAGGUUCGUUGGGCTT-3’</td>
</tr>
<tr>
<td>ATF-1 siRNA - sense</td>
<td>5’-GC GGACAGUACAUUGCCAUTT-3’</td>
</tr>
<tr>
<td>ATF-1 siRNA - antisense</td>
<td>5’-AUGGCAAUGUACUGUCGCCTT-3’</td>
</tr>
<tr>
<td>Scrambled ATF-1 siRNA - sense</td>
<td>5’-GCGUGACUACACGUAGCAUTT-3’</td>
</tr>
<tr>
<td>Scrambled ATF-1 siRNA - antisense</td>
<td>5’-AUGCUACGUGUAGUCACGCTT-3’</td>
</tr>
</tbody>
</table>

**Metalloproteinase Activity.** Two fluorogenic peptide substrates from R&D Systems (Minneapolis, MN), Substrate III and IX, were used to measure metalloproteinase activity. These peptide substrates contain a highly fluorescent 7-methoxycoumarin group (Mca) that is efficiently quenched by resonance energy transfer to the 2,4-dinitrophenyl group (Dpa). They can be used to measure the activities of peptidases that are capable of cleaving an amide bond between the fluorescent group and the quencher group, causing an increase in fluorescence. Substrate III (Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH2) is a substrate for tumor necrosis factor-α converting enzyme (TACE or ADAM17) and related enzymes such as ADAM9 and ADAM10. Substrate IX (Mca-K-P-L-G-L-Dpa-A-R-NH2) is a substrate for all matrix metalloproteinases (MMPs). Medial smooth muscle cells were plated on six-well plates and cultured for 24 h in normal growth medium (at approximately 80% confluence), then serum-deprived (0.2% FBS) for another 24 h before stimulation with Eh Cys/CySS redox medium. Prior to treatment, fluorogenic substrates were added to the control or Eh=0 mV medium in the final concentration of 10 µM and SMCs were then incubated for various amount of time. At the end of each incubation period, the media were collected and the fluorescence was measured.
using SpectraMax M2 fluorescence plate reader (Molecular Devices, Sunnyvale, CA), with the following settings: excitation wavelength 320 nm, emission wavelength 405 nm. An appropriate blank (without fluorogenic substrate) was subtracted from each measurement and the results were expressed as the fold difference over control values and normalized to cell number.

**Gelatin Zymography.** Latent and activated forms of MMP-2 and MMP-9 were detected using SDS-PAGE gelatin zymography, as described previously. Medial smooth muscle cells were cultured on six-well plates for 24 h in normal growth medium at approximately 80% confluence, then serum-deprived (0.2% FBS) for another 24 h before stimulation with \( E_h \) Cys/CySS redox medium for different periods of time. After treatment, the conditioned media were collected and centrifuged at 13,000 g for 5 min at 4°C to remove cell debris. The samples were then concentrated using Millipore Centricon tubes, according to the manufacturer’s recommendation. Concentrated samples were mixed with non-reducing sample buffer and electrophoretically separated on 10% Bio-Rad Ready Gel zymogram gels containing 1 mg/ml gelatin. Following electrophoresis, gels were soaked in NOVEX Zymogram renaturing buffer (Invitrogen, Carlsbad, CA) for 1 h at room temperature to remove SDS, and then incubated in NOVEX Zymogram developing buffer at 37°C for 48 h, to allow enough time for the metalloproteinases to digest gelatin. Sample bands with proteolytic activity were visualized after staining with Coomassie Brilliant Blue (CBB) R-250 (0.25% CBB R-250 in 40% methanol and 10% acetic acid) as clear bands against a dark blue background. After gel staining, MMP-2 and MMP-9 were identified based on gelatin lysis at molecular masses of 62 kDa for MMP-2 and 84 kDa for MMP-9, whereas pro-MMP-2 and pro-MMP-9 were identified at 72 kDa and 92 kDa, respectively. Gel images were obtained using a digital imaging system from Eastman Kodak (Rochester, NY).
**Luciferase Assay for Promoter Activity.** pGL3-basic (empty vector) or Nox1 promoter-luciferase plasmids (gift from Dr. Chihiro Yabe-Nishimura, Kyoto Prefectural University of Medicine, Japan) were co-transfected into SMCs with pSV40-Renilla control vectors. Medial smooth muscle cells were plated on twelve-well plates and cultured for 24 h in normal growth medium (at approximately 70% confluence). Next, pGL3-basic (empty vector) or Nox1 promoter-luciferase plasmids were co-transfected into SMCs (750 ng/well) with pSV40-Renilla control vectors (5 ng/well) using Lipofectamine LTX and PLUS Reagent (Invitrogen) in OPTI-MEM I reduced serum medium. pSV40-Renilla plasmid served as a control for transfection. After 4 h of transfection, SMCs were cultured for 24 h in a normal growth medium then serum-deprived (0.2% FBS) for another 24 h before incubation with various $E_b$ Cys/CySS redox medium. After treatment for 8 h, cells were lysed and luciferase activity measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Results were expressed as a ratio between the firefly and Renilla luciferase.

**Statistical Analysis.** Results are expressed as mean ± SEM. Statistical comparisons were performed by Student’s two-tailed $t$-test or one-way analysis of variance (ANOVA) followed by Dunnett’s or Tukey’s multiple comparison post-tests, where appropriate. A value of $p < 0.05$ was considered significant.
References


Supplemental Figure I. Oxidized extracellular $E_h$ increases Nox1 expression in vascular SMCs, but not in HEK 293 and Caco2 cells. Cells were exposed to varying concentrations of Cys and CySS to obtain a range of $E_h$ from -150 mV to 0 mV for 24 hours. (A) SMCs were infected with adenovirus encoding siNox1 (Ad-siNox1) or with Ad-siGFP as a control for three days prior to exposure to -150 mV or 0 mV Cys/CySS redox conditions. Nox1 protein expression was measured by Western blotting. (B) Summary data from four independent experiments. Results
are normalized to GAPDH expression (mean ± SE, * p < 0.05 vs. -150 mV, Δp < 0.05 vs. 0 mV Ad-siGFP). Nox1 (C, E) and Nox4 (D) mRNA expression was measured by qRT-PCR. The data are presented as mean ± SE (n=4 to 5). * p < 0.05 vs. -150 mV or control.
Supplemental Figure II. AG1478 prevents EGFR phosphorylation by an oxidized extracellular \( E_h \). (A) SMCs were treated with the control medium and p-EGFR expression analyzed by Western blotting. Results are normalized to total EGFR expression (n=3). (B) SMCs were pretreated with 10 μM AG1478, then exposed to \( E_h =0 \) mV for 1 min. Phospho-EGFR expression was analyzed by Western blotting. Results are normalized to total EGFR expression and presented as mean ± SE (n=5). * \( p < 0.05 \) vs. control; Δ \( p < 0.05 \) vs. 0 mV vehicle.
**Supplemental Figure III.** Oxidized extracellular $E_h$ differentially activates EGFR downstream signaling targets. SMCs were exposed to $E_h=0 \text{ mV}$ for indicated amount of time and p-ERK1/2 (A), p-Akt (B), p-p38 (C), and p-JNK (D) were analyzed by Western blotting. Results are normalized to total ERK (A) or GAPDH expression (B, C, D) and presented as mean ± SE (n=3 to 4). Blots shown were run on the same gel but cut for better presentation. * $p < 0.05$ vs. untreated (0 min).
Supplemental Figure IV. Nox1 expression is increased via the MEK pathway. SMCs were infected with adenovirus encoding constitutively active form of MEK (Ad-caMEK) or with Ad-empty as a control. (A) Representative blots show MEK and p-ERK1/2 expression, respectively. GAPDH served as a loading control (n=3). (B) Nox1 mRNA expression was measured by qRT-PCR (n=4). (C) SMCs were transfected with Nox1 promoter luciferase or control plasmid and infected with Ad-caMEK or Ad-empty. Firefly luciferase activity was normalized to Renilla luciferase. Data are presented as mean ± SE (n=5). * p < 0.05 vs. Ad-empty.
Supplemental Figure V. siRNAs to CREB and ATF-1 are effective and specific for their target. SMCs were transfected with 50 nM siRNA to CREB or ATF-1, then exposed to $E_\text{h}=0$ mV for 5 min. (A) p-CREB and p-ATF-1 expression were analyzed by Western blotting. Scrambled siRNA served as a control. Results are normalized to GAPDH expression. (B) Summary data of n=5 independent experiments. * $p < 0.05$ vs. control; $\Delta p < 0.05$ vs. 0 mV control siRNA.
Supplemental Figure VI. ATF-1, but not CREB, is activated via the metalloproteinase-EGFR-ERK1/2 pathway in response to oxidized extracellular Eₘ. SMCs were pretreated with GM6001 (A), 10 μM AG1478 (B) or 10 μM U0126 and 1 μM wortmannin (C) and stimulated with Eₘ=0 mV for 5 min. Representative blots show p-CREB and p-ATF-1 expression. Blots shown in A and in B were run on the same gel but cut for better presentation. Summary data of four independent experiments are shown (n=3 to 5). GAPDH served as a loading control (mean ± SE, * p < 0.05 vs. control; Δ p < 0.05 vs. 0 mV vehicle).
Supplemental Figure VII. Summary of signaling pathways involved in oxidized extracellular 
E$_h$-mediated activation of Nox1 expression. Extracellular oxidative stress, involving oxidation 
of the Cys/CySS redox couple, activates the EGFR through metalloproteinase-mediated shedding 
of EGF-like ligands, leading to ERK1/2-dependent activation of the transcription factor ATF-1 
and subsequent expression of Nox1. Generation of ROS by activation of Nox1 promotes 
phenotypic modifications in SMCs, such as cell proliferation, that contribute to vascular disease.