Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression

Xi-Lin Chen, Geraldine Dodd, Suzanne Thomas, Xiaolan Zhang, Martin A. Wasserman, Brad H. Rovin, and Charles Kunsch

1Discovery Research, AtheroGenics, Incorporated, Alpharetta, Georgia; and 2Department of Internal Medicine and Dorothy M. Davis Heart and Lung Research Institute, Ohio State University College of Medicine and Public Health, Columbus, Ohio

Submitted 16 June 2005; accepted in final form 30 November 2005

Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. Am J Physiol Heart Circ Physiol 290: H1862–H1870, 2006. First published December 9, 2005; doi:10.1152/ajpheart.00651.2005.—The antioxidant response element (ARE) is a transcriptional control element that mediates expression of a set of antioxidant proteins. NF-E2-related factor 2 (Nrf2) is a transcription factor that activates ARE-containing genes. In endothelial cells, the ARE-regulated genes are upregulated by atheroprotective laminar flow through a Nrf2-dependent mechanism. We tested the hypothesis that activation of ARE-regulated genes via adenovirus-mediated expression of Nrf2 may suppress redox-sensitive inflammatory gene expression. Expression of Nrf2 in human aortic endothelial cells (HAECs) resulted in a marked increase in ARE-driven transcriptional activity and protected HAECs from H2O2-mediated cytotoxicity. Nrf2 suppressed TNF-α-induced monocyte chemoattractant protein (MCP)-1 and VCAM-1 mRNA and protein expression in a dose-dependent manner and inhibited TNF-α-induced monocyte U937 cell adhesion to HAECs. Nrf2 also inhibited IL-1β-induced MCP-1 gene expression in human mesangial cells. Expression of Nrf2 inhibited TNF-α-induced activation of p38 MAP kinase. Furthermore, expression of a constitutively active form of MKK6 (an upstream kinase for p38 MAP kinase) partially reversed Nrf2-mediated inhibition of VCAM-1 expression, suggesting that p38 MAP kinase, at least in part, mediates Nrf2's anti-inflammatory action. In contrast, Nrf2 did not inhibit TNF-α-induced NF-κB activation. These data identify the Nrf2/ARE pathway as an endogenous atheroprotective system for antioxidant protection and suppression of redox-sensitive inflammatory genes, suggesting that targeting the Nrf2/ARE pathway may represent a novel therapeutic approach for the treatment of inflammatory diseases such as atherosclerosis.

THE ANTIOXIDANT RESPONSE ELEMENT (ARE), also referred to as the electrophile response element, is a cis-acting transcriptional regulatory element involved in the activation of genes coding for a number of antioxidant proteins and phase II detoxifying enzymes including NADPH:quinone oxidoreductase (NQO1), glutathione peroxidase (GPx), ferritin, and heme oxygenase-1 (HO-1) (11, 32). NF-E2-related factor 2 (Nrf2) is the transcription factor that is responsible for both constitutive and inducible expression of the ARE-regulated genes (5, 19). Nrf2-null mice have decreased basal and inducible expression of antioxidant genes, increased oxidative stress, and decreased reducing activity and antioxidant capacity (4, 14), suggesting that the Nrf2/ARE pathway is critical for the regulation of intracellular redox status. In addition to its role in regulating redox levels, several recent studies using Nrf2-deficient mice have demonstrated that the Nrf2/ARE pathway is involved in immune and inflammatory processes. Yoh and coworkers (45) reported that aged female Nrf2-deficient mice developed lupus-like nephritis. Furthermore, it was demonstrated that Nrf2-deficient mice exhibit prolonged inflammation during cutaneous wound healing (3) and display enhanced bronchial inflammation and susceptibility to cigarette smoke-induced emphysema (33). These data suggest that Nrf2 deficiency and the resulting impaired antioxidant activity are important in the determination of susceptibility to autoimmune and inflammatory diseases.

It is now well established that atherosclerosis is an inflammatory disease (27). Initial atherosclerotic lesions preferentially develop in areas of the vasculature exposed to low fluid shear stress and nonlaminar blood flow. Conversely, lesion development is decreased in regions exposed to high fluid shear stress and steady laminar flow (2). In our earlier studies (8), endothelial cells subjected to prolonged physiological levels of laminar shear stress had increased expression of ARE-mediated antioxidant genes such as NQO1, HO-1, and ferritin through a Nrf2-dependent mechanism.

The inducible expression of several inflammatory genes, including VCAM-1 and monocyte chemoattractant protein (MCP)-1, is known to be regulated through oxidation-reduction (redox)-sensitive mechanisms and plays a crucial role in the initiation and progression of atherosclerosis (27, 28). Because oxidant stress contributes to the pathogenesis of atherosclerosis by stimulating inflammatory gene expression, we hypothesized that the enhanced expression of the Nrf2/ARE-regulated cytoprotective proteins may contribute to the atheroprotective and anti-inflammatory phenotype observed in areas of the vasculature exposed to laminar flow. Therefore, we tested the hypothesis that activation of the Nrf2/ARE pathway may suppress redox-sensitive inflammatory gene expression and protect against oxidant-mediated injury in endothelial cells.

METHODS

Cell culture. Human aortic endothelial cells (HAECs) were obtained from Cambrex Bio Science and cultured in endothelial growth medium (EGM)-2. Cells were used between passages 5 and 9. Human dermal microvascular endothelial cells (HMECs) were described...
previously (7) and were cultured in modified MCDB 131 (Invitrogen) supplemented with EGM SingleQuot (Cambrex Bio Science). Human mesangial cells were described previously (34) and were grown in RPMI 1640 containing 10% fetal bovine serum.

Materials. p3xARE/Luc and LNCX-Nrf2 were described previously (8). 5xNF-κB/Luc was purchased from Promega. Antibodies for phosphorylated p38 MAP kinase and phosphorylated JNK were obtained from Promega. Antibodies for p38 MAP kinase, JNK, and HO-1 were obtained from Cell Signaling Technology. Antibodies for Nrf2 and IκB-α were purchased from Santa Cruz Biotechnology. Antibody for GPx was obtained from Lab Frontier Science Institute (Seoul, Korea).

Adenoviruses. The adenovirus encoding murine Nrf2 cDNA (Ad.Nrf2) was previously described (46). Ad.DN-Nrf2, encoding a dominant-negative murine Nrf2 gene, was generated by cloning the COOH-terminal amino acids 399–589 of Nrf2 into the shuttle vector pVQ Ad5CMV K-NpA (ViralQuest). Ad.MKK6(E), a constitutively active form of MKK6, was kindly provided by Dr. J. Han (Department of Immunology, Scripps Research Institute, La Jolla, CA) (40). Ad.GFP, an adenovirus encoding the green fluorescent protein (GFP) gene, was used as a control for adenovirus infection.

Determination of intracellular GSH levels. Confluent HAECs in 10-cm dishes were either mock infected or infected with Ad.GFP or Ad.Nrf2 at a multiplicity of infection (MOI) of 100 for 24 h, and cells were lysed in 5% meta-phosphoric acid and homogenized by passing through a 26-gauge needle. Homogenates were centrifuged at 3,000 g for 10 min, and 300 μl of resulting supernatant was used for assay. GSH levels in samples were measured with a glutathione assay kit from EMD Biosciences (La Jolla, CA). Absorbance was measured at 400 nm with the Visible Spectrophotometer from Shimadzu (UV-1606). A standard curve of known GSH concentrations plotted against measured absorbance was used to determine GSH concentrations of samples.

Determination of cytotoxicity through assay of lactate dehydrogenase. Cytotoxicity was determined by measuring the levels of lactate dehydrogenase (LDH) in the medium with a CytoTox 96 nonradioactive cytotoxity assay (EMD Biosciences).

Analysis of mRNA levels. Total RNA samples were isolated and quantitatively measured by UV spectroscopy. VCAM-1, MCP-1, and GAPDH mRNA levels were determined with a Quantikine mRNA colorimetric quantification kit (R&D Systems) according to the manufacturer’s instructions.

ELISA for MCP-1 and VCAM-1 protein. HAECs growing in 24-well plates were treated with TNF-α (100 U/ml) for 16 h. Conditioned media were collected and assayed for MCP-1 protein levels by ELISA, using a Quantikine colorimetric sandwich ELISA kit (R&D Systems) according to the manufacturer’s instructions. Cell surface expression of VCAM-1 was determined as previously described (5).

Monocyte adhesion assay. The human monocytic cell line U937 was used to evaluate endothelial cell-monocyte adhesion and was described previously (24). Briefly, the fluorescently labeled U937 cell suspension was added to the HAEC monolayers in 24-well plates, and the mixture was incubated at 37°C for 30 min. Adherent U937 cells were removed by adding 200 μl of 1 mM EDTA-PBS per well and incubating at room temperature for 30 min. Plates were visualized using fluorescence microscopy to determine the number of adherent U937 cells. The monocyte-containing EDTA-PBS solution was mixed thoroughly by pipetting and transferred to a 96-well plate. Fluorescence was measured with the PerkinElmer Victor2 V multiplate reader.

Transfection and promoter activity assays. Because HAECs are relatively resistant to efficient transient transfection, we used HMECs for these experiments. HMECs were transfected with various plasmids by using SuperFect transfection reagent (Qiagen). Luciferase activities were measured with a luciferase reporter assay system (Promega).

Western blot analysis. Protein samples were subjected to electrophoresis on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Antibody-bound protein bands were then visualized by horseradish peroxidase-dependent chemiluminescence (Amersham).

ELISA for nuclear NF-κB and activator protein-1 binding activities and phosphorylated and total p38 MAP kinase levels. HAEC nuclear extracts were prepared as previously described (39). Nuclear NF-κB or activator protein-1 (AP-1) binding activity was determined by using TransAM transcriptional factor assaying kits for NF-κB p65 or AP-1 c-Jun according to the manufacturer’s instructions (Active Motif, North American). This is an ELISA-based quantitative assay of nuclear NF-κB or AP-1 binding activity using antibodies directed to NF-κB p65 subunit or phosphorylated c-Jun of the AP-1 family. Phosphorylated and total p38 MAP kinase levels were determined by ELISA (BioSource, Camarillo, CA) according to the manufacturer’s instructions.

Statistical analysis. Values are expressed as means ± SD of at least three independent experiments. Statistics were performed by ANOVA and followed with Tukey, and values were considered significantly different at the 95% confidence level.

RESULTS

Functional characterization of adenovirus-mediated expression of Nrf2 and activation of ARE pathway in endothelial cells. By Western blot analysis, infection of HAECs with Ad.Nrf2 resulted in a marked increase in intracellular Nrf2 protein level compared with HAECs that were mock infected or infected with Ad.GFP (Fig. 1A, top). Expression of Nrf2 also produced a marked increase in HO-1 and GPx protein levels (Fig. 1A, middle); both were regulated by the Nrf2/ARE pathway (6, 38). In contrast, expression of Nrf2 had no effect on protein levels of Ca/Zn-SOD, a non-ARE-regulated gene (Fig. 1A, bottom). Furthermore, infection with Ad.Nrf2 resulted in a dose-dependent increase in ARE-driven transcriptional activity in HMECs (Fig. 1B). Similar to HAECs, expression of Nrf2 also increased HO-1 protein levels in HMECs (data not shown).

Expression of Nrf2 increases intracellular GSH levels and protects endothelial cells from H2O2-mediated cytotoxicity. GSH is an abundant intracellular thiol that serves to buffer changes in the cellular redox status, reduces peroxides, and participates in detoxification of reactive oxygen species. The rate-limiting step in GSH biosynthesis is mediated by γ-glutamylcysteine synthetase (γ-GCS), which is upregulated by Nrf2 (41). To determine whether expression of Nrf2 modulates intracellular redox balance in endothelial cells, HAECs were mock infected or infected with Ad.Nrf2 or Ad.GFP for 24 h and GSH levels were measured. Infection with Ad.Nrf2 resulted in an ~25% increase in intracellular GSH levels compared with mock-infected or Ad.GFP-infected cells (Fig. 2A).

To investigate the effects of activation of the ARE pathway on oxidative stress-induced endothelial cell injury, HAECs were infected with Ad.Nrf2 or Ad.GFP and then exposed to H2O2. Treatment of Ad.GFP-infected cells with H2O2 resulted in a marked increase in cytotoxicity as determined by release of LDH from the cells. Expression of Nrf2 produced a ~50% suppression of H2O2-induced cytotoxicity (Fig. 2B). These data suggest that activation of the Nrf2/ARE pathway increases intracellular antioxidant capacity and protects endothelial cells from oxidative stress-mediated cell injury.

Expression of Nrf2 inhibits cytokine-induced MCP-1 expression in endothelial and mesangial cells. To investigate the effects of expression of Nrf2 on MCP-1 expression, HAECs were mock infected or infected with Ad.GFP or Ad.Nrf2 and...
Nrf2 was accompanied by a similar decrease in MCP-1 mRNA expression by \( \sim 63\% \), relative to cells treated with IL-1\(\beta\) plus Ad.GFP (data not shown).

\textit{Ad.Nrf2 suppresses TNF-\(\alpha\)-induced cell surface expression of VCAM-1 protein and mRNA accumulation.} We further determined the effect of expression of Nrf2 on the expression of the redox-sensitive gene VCAM-1. As shown in Fig. 4A, the TNF-\(\alpha\)-induced increase in VCAM-1 mRNA levels was inhibited by Ad.Nrf2 by \( \sim 80\% \) compared with cells infected with Ad.GFP. Expression of Nrf2 also produced a dose-dependent inhibition of cell surface expression of VCAM-1 stimulated by TNF-\(\alpha\) (Fig. 4B), whereas infection with Ad.GFP had no effect. These data suggest that activation of the Nrf2/ARE pathway suppresses cytokine-induced expression of the redox-sensitive genes VCAM-1 and MCP-1.

\textit{Expression of Nrf2 suppresses TNF-\(\alpha\)-induced monocytic cell adherence to endothelial cells.} VCAM-1 is a primary adhesion molecule that mediates the attachment of leukocytes to the endothelium. Because expression of Nrf2 inhibits TNF-\(\alpha\)-induced VCAM-1 expression, we examined whether this would translate into reduced monocyte adhesion to endothelial cells. As shown in Fig. 5, TNF-\(\alpha\) treatment of endothelial cells induced a marked increase in U937 adherence to HAECSs in both mock- and Ad.GFP-infected cells. Expression of Nrf2 resulted in an \( \sim 80\% \) inhibition of TNF-\(\alpha\)-induced U937 adhesion to endothelial cells compared with Ad.GFP-infected cells. These data suggest that activation of the Nrf2/ARE...
pathway in vivo may suppress leukocyte infiltration into inflamed tissues.

**Dominant-negative Nrf2 has no effect on TNF-α-induced VCAM-1 and MCP-1 expression.** Recent studies show that Nrf2-deficient mice have increased pulmonary inflammatory responses. To investigate the effects of inhibition of Nrf2 function on TNF-α-induced VCAM-1 and MCP-1 expression, we used a dominant-negative Nrf2 adenovirus. Expression of dominant-negative Nrf2 led to an increased expression of truncated Nrf2 protein and suppressed Nrf2-induced ARE-driven promoter activity but did not inhibit basal ARE promoter activity in endothelial cells (Fig. 6A). Interestingly, expression of dominant-negative Nrf2 did not affect TNF-α-induced VCAM-1 and MCP-1 expression (Fig. 6, B and C).

**Expression of Nrf2 has no effect on NF-κB activation.** We examined the effect of Nrf2 on TNF-α-induced NF-κB activation in endothelial cells through three approaches: nuclear NF-κB binding activity, IkB-α degradation, and NF-κB promoter activity. As expected, TNF-α induced nuclear NF-κB binding activity and IkB-α degradation in Ad.GFP-infected HAECS. Expression of Nrf2 did not suppress TNF-α-induced degradation of IkB-α (Fig. 7A) and NF-κB nuclear binding activity (Fig. 7B) in HAECS. Similarly, expression of Nrf2 did not inhibit TNF-α-induced NF-κB-driven promoter activity in HMECs (Fig. 7C). These data suggest that inhibition of MCP-1 and VCAM-1 gene expression by Nrf2 is not due to inhibition of NF-κB.

**Expression of Nrf2 does not suppress TNF-α-induced AP-1 activation.** AP-1 regulates many immune and inflammatory genes including VCAM-1 and MCP-1 (1, 13). Infection of HAECS with Ad.GFP led to an increase in nuclear AP-1 binding activity compared with mock-infected HAECS. Expression of Nrf2 by itself further increased nuclear AP-1 binding activity compared with Ad.GFP-infected cells. Treatment of Ad.GFP-infected HAECS with TNF-α-induced nuclear

---

**Fig. 3.** Expression of Nrf2 suppresses TNF-α-induced monocyte chemoattractant protein (MCP)-1 expression. HAECS were infected with Ad.GFP (MOI of 100) or Ad.Nrf2 (MOI of 25, 50, and 100) for 24 h and then exposed to TNF-α (100 U/ml) for 4 h. A: MCP-1 mRNA levels were determined and normalized to GAPDH levels. B: conditioned medium was collected, and MCP-1 protein levels were determined. Results are expressed as fold change over Ad.GFP-transfected cells. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α-treated cells infected with Ad.GFP.

**Fig. 4.** Expression of Nrf2 suppresses TNF-α-induced VCAM-1 expression. HAECS were infected with Ad.GFP (MOI of 100) or Ad.Nrf2 (MOI of 25, 50, and 100) for 24 h and then exposed to TNF-α (100 U/ml) for 4 h. A: VCAM-1 mRNA levels were determined and normalized to GAPDH levels. B: cell surface expression of VCAM-1 was determined by ELISA. Results are expressed as fold change over Ad.GFP-transfected cells. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α-treated cells infected with Ad.GFP.

**Fig. 5.** Expression of Nrf2 suppresses TNF-α-induced monocytic U937 cell adhesion to HAECS. HAECS grown in 24-well plates were infected with Ad.GFP or Ad.Nrf2 (MOI of 100) for 24 h and then exposed to TNF-α (100 U/ml) for 4 h. Adhesion of monocytic cell line U937 cells was determined. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α-treated cells infected with Ad.GFP.
AP-1 binding activity at 30 min and 1 h. Expression of Nrf2 did not suppress TNF-α-induced AP-1 nuclear binding activity (data not shown). These data suggest that inhibition of MCP-1 and VCAM-1 gene expression by Nrf2 is not due to inhibition of AP-1.

Expression of Nrf2 suppresses TNF-α-induced activation of p38, but not JNK, MAP kinase in endothelial cells. p38 and JNK MAP kinases are involved in TNF-α-induced inflammatory gene expression (35, 44). TNF-α treatment resulted in a marked increase in phosphorylated p38 and JNK MAP kinase levels in Ad.GFP-infected cells. Expression of Nrf2 inhibited TNF-α-induced activation of p38 MAP kinase. In contrast, Ad.Nrf2 by itself slightly increased JNK activity and had little or no effect on JNK activation (Fig. 8). Infection with either Ad.GFP or Ad.Nrf2 had no effect on total protein levels of p38.

To further investigate the role of p38 MAP kinase in Nrf2’s action, we examined the effects of either pharmacological inhibition of p38 MAP kinase or constitutive activation of p38 MAP kinase on Nrf2-mediated inhibition of inflammatory gene expression. We overexpressed a constitutively active form of MKK6 (upstream kinase that directly activates p38 MAP kinase) with Ad.MKK6(E). As expected, infection with

Fig. 6. Dominant-negative (DN) Nrf2 has no effect on TNF-α-induced VCAM-1 and MCP-1 expression. A: HMECs were transfected with 1 μg of 3xARE-luc and 0.1 μg of pRL-SV40 for normalization of transfection efficiency. Cells were then mock infected or infected with Ad.GFP (MOI of 200), Ad.DN-Nrf2 (MOI of 200), Ad.Nrf2 (MOI of 100), or both Ad.DN-Nrf2 (MOI of 200) and Ad.Nrf2 (MOI of 100) for 24 h. Cell extracts were harvested, and luciferase assays were performed. B: HAECs were infected with Ad.GFP or Ad.DN-Nrf2 (MOI of 200) for 24 h and then exposed to TNF-α (100 U/ml) for 4 h. Cell surface expression of VCAM-1 was determined. OD, optical density. C: conditioned medium was collected, and MCP-1 protein levels were determined. Values represent means ± SD; n = 4. *P < 0.05 compared with Ad.Nrf2-infected cells.

Fig. 7. Expression of Nrf2 has no effect on TNF-α-induced activation of NF-κB. A: HAECs infected with Ad.GFP or Ad.Nrf2 (MOI of 100) for 24 h were exposed to TNF-α (100 U/ml) for 1 h. Whole cell lysates were analyzed by immunoblotting with antibody to IκB-α or β-tubulin. B: HAECs were infected with Ad.GFP or Ad.Nrf2 (MOI of 100) for 24 h and then exposed to TNF-α (100 U/ml) for 1 h. Nuclear extracts were isolated, and nuclear NF-κB binding activity was determined with a TransAM NF-κB p65 transcription factor kit. Values are means ± SD; n = 4. C: HMECs cultured in 12-well plates were transfected with 1 μg 5xNFκB/Luc + 1 μg LNCX-Nrf2 or empty vector LNCX. After a 24 h recovery, cells were exposed to TNF-α (100 U/ml) for 16 h, cell extracts were harvested, and luciferase assays were performed. The firefly luciferase activities were normalized by Renilla luciferase activities. Values represent means ± SD; n = 4.
Ad.MKK6(E) resulted in an increase in MKK6 protein levels in HAECs (Fig. 9A). Infection with Ad.MKK6(E) had no effect on TNF-\(\alpha\)/H9251-induced VCAM-1 expression but partially reversed the Ad.Nrf2-mediated inhibition of TNF-\(\alpha\)/H9251-induced VCAM-1 expression (Fig. 9B). In contrast, treatment of HAECs with the p38 MAP kinase inhibitor SB-202190 (5 \(\mu\)M) resulted in 27% inhibition of TNF-\(\alpha\)/H9251-induced VCAM-1 expression. Infection with Ad.Nrf2 (MOI of 50) resulted in a 60% inhibition of TNF-\(\alpha\)/H9251-induced VCAM-1 expression. The combination of Ad.Nrf2 and SB-202190 led to 96% inhibition of VCAM-1 expression (Fig. 9C). These data suggest that Nrf2-mediated inhibition of inflammatory expression is, at least in part, mediated by suppression of p38 MAP kinase activation.

**DISCUSSION**

Oxidative signals play important roles in the pathogenesis of chronic inflammatory diseases by mediating expression of inflammatory genes. However, the protective mechanisms of antioxidant systems remain poorly defined. The Nrf2/ARE pathway may serve as an endogenous antioxidant system within the vasculature that is activated by atheroprotective laminar flow (8). In the present study, expression of Nrf2 in endothelial cells resulted in a marked increase in ARE-driven transcriptional activity, upregulation of two ARE-driven antioxidant proteins (HO-1 and GPx), increased intracellular GSH levels, and protection from oxidant-mediated injury. Furthermore, expression of Nrf2 suppressed TNF-\(\alpha\)-induced MCP-1 and VCAM-1 expression, monocyte adhesion to endothelial cells, and activation of p38 MAP kinase. Cumulatively, these observations provide the first demonstration of a role for the Nrf2/ARE pathway in modulating redox-sensitive inflammatory gene expression in endothelial cells and suggest a function for this pathway in regulating chronic inflammatory diseases of the vasculature.

It is well established that physiological levels of laminar shear stress exert anti-inflammatory and atheroprotective effects (2). However, the underlying molecular mechanisms are still not fully understood. Laminar shear stress may prevent atherosclerosis by increasing intracellular antioxidant capacity.
and suppressing redox-sensitive inflammatory genes such as VCAM-1 and MCP-1. Exposure of endothelial cells to laminar shear stress upregulates the antioxidant proteins Mn-SOD, Cu/Zn-SOD, HO-1, and endothelial nitric oxide synthase. In a previous study, we demonstrated (8) that laminar shear stress activates ARE-mediated transcriptional activity and increases expression of a set of antioxidant genes such as NQO1, HO-1, γ-GCS, and ferritins through Nrf2-dependent mechanisms. In the present study, we mimicked one physiological component of laminar shear stress by activation of the Nrf2/ARE pathway. Infection of endothelial cells with Ad.Nrf2 produced an eight-fold increase in ARE-driven promoter activity, which is comparable with the level of ARE promoter activity induced by laminar flow in our earlier study (8). Overexpression of Nrf2 also resulted in an upregulation of the antioxidant proteins HO-1 and GPx and elevated the intracellular levels of GSH. This latter observation is likely due to Nrf2-mediated expression of γ-GCS. These results suggest that the Nrf2/ARE pathway may, at least in part, be involved in the laminar shear stress-mediated atheroprotective and anti-inflammatory effects in vasculature.

Through promoter analysis and DNA microarray analysis of Nrf2-deficient mice, numerous studies have shown that the Nrf2/ARE pathway regulates several clusters of genes that are important in detoxification and antioxidant (6, 26). The Nrf2/ARE pathway is critically important in regulation of cellular redox balance by modulating genes involved in several antioxidant systems. γ-GCS, GPx, glutathione reductase, malic enzyme 1, thioredoxin reductase, thioredoxin, peroxiredoxin MSPl, and the cysteine/glutamate exchange transport system are all regulated via the Nrf2/ARE pathway and act directly to replenish the cell’s major reductants (16, 36). Gluthione reductase is activated by laminar shear stress and inhibits H2O2-mediated activation of JNK (15). Thioredoxin is an important intracellular antioxidant that protects cells from oxidative stress and inhibits inflammatory signaling (43). Our data showing that expression of Nrf2 increases intracellular GSH levels in endothelial cells are consistent with the role of Nrf2 in the activation of genes involved in glutathione biosynthesis and reduction. Nrf2 also regulates a set of antioxidant proteins such as HO-1, ferritin, peroxiredoxin, and metallothio-nein (6, 26). The coordinated regulation of these genes can have synergistic effects on the maintenance of intracellular antioxidant capacity, protection of endothelial cells from oxidant-mediated injury, and inhibition of inflammatory responses. Studies have shown that HO-1 plays an important role in modulating inflammatory responses. Targeted deletion of HO-1 resulted in chronic inflammation characterized by hepatosplenomegaly, leukocytosis, glomerulonephritis, and hepatic peribronchial inflammation (31). Conversely, expression of HO-1 protected against oxidant and inflammatory injuries in several animal models. Targeted expression of HO-1 prevented pulmonary inflammatory and vascular responses to hypoxia (29). Overexpression of HO-1 inhibited atherosclerotic lesion formation in LDL-receptor knockout mice and in Watanabe heritable hyperlipidemic rabbits (17). Also, induction of HO-1 in vascular cells suppressed oxidized LDL-induced monocyte transmigration (17, 18) and inhibited cytokine-induced VCAM-1 and MCP-1 (22, 37). HO-1 overexpression also exerted beneficial effects in a number of transplantation mod-
may way may serve as a new therapeutic approach for the treatment of inflammatory diseases such as atherosclerosis.

REFERENCES


4. Chan JY and Kwong M. Impaired expression of glutatione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochim Biophys Acta 1517: 19–26, 2000.


33. Tummala PE, Chen XL, and Medford RM. NF-κB independent suppression of endothelial vascular cell adhesion molecule-1 and intercellular


