Activation of Nrf2 in Endothelial Cells Protects Arteries From Exhibiting a Proinflammatory State

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Objective—Proinflammatory mediators influence atherosclerosis by inducing adhesion molecules (eg, VCAM-1) on endothelial cells (ECs) via signaling intermediaries including p38 MAP kinase. Regions of arteries exposed to high shear stress are protected from inflammation and atherosclerosis, whereas low-shear regions are susceptible. Here we investigated whether the transcription factor Nrf2 regulates EC activation in arteries.

Methods and Results—En face staining revealed that Nrf2 was activated in ECs at an atheroprotected region of the murine aorta where it negatively regulated p38–VCAM-1 signaling, but was expressed in an inactive form in ECs at an atherosusceptible site. Treatment with sulforaphane, a dietary antioxidant, activated Nrf2 and suppressed p38–VCAM-1 signaling at the susceptible site in wild-type but not Nrf2−/− animals, indicating that it suppresses EC activation via Nrf2. Studies of cultured ECs revealed that Nrf2 inactivates p38 by suppressing an upstream activator MKK3/6 and by enhancing the activity of the negative regulator MKP-1.

Conclusions—Nrf2 prevents ECs at the atheroprotected site from exhibiting a proinflammatory state via the suppression of p38–VCAM-1 signaling. Pharmacological activation of Nrf2 reduces EC activation at atherosusceptible sites and may provide a novel therapeutic strategy to prevent or reduce atherosclerosis. (Arterioscler Thromb Vasc Biol. 2009; 29:1851-1857.)

Key Words: Nrf2 ■ arterial endothelium ■ shear stress ■ sulforaphane ■ proinflammatory activation ■ p38 ■ MKK3/6 ■ MKP-1

Early atherosclerotic lesions contain monocytes and T-lymphocytes which are recruited from the circulation by adhesion to activated vascular endothelial cells (ECs).1 This process is triggered by proinflammatory mediators (eg, TNFα) which induce cellular adhesion molecules (eg, VCAM-1) via signaling intermediaries including p38 mitogen-activated protein (MAP) kinase, which is activated by phosphorylation by MAP kinase kinases 3 and 6 (MKK 3/6).2,3 Vascular inflammation and atherosclerosis develop predominantly at distinct sites of the arterial tree located near branches and bends which are exposed to nonuniform blood flow, which exerts relatively low shear stress on vascular endothelium, whereas regions of arteries that are exposed to unidirectional high shear stress are protected.4–6 Proinflammatory activation of ECs is reduced at high-shear sites compared to low-shear regions, thus providing a potential explanation for the distinct spatial localization of vascular inflammation and lesion formation.5,7–10 Similarly, the application of unidirectional high shear stress can suppress proinflammatory activation of cultured ECs, whereas low or oscillatory shear can act as a positive regulator of EC activation.5,10–15

The molecular mechanisms underlying the antiinflammatory effects of shear stress are uncertain, but previous studies of cultured cells have suggested a role for the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2).16–20 In unstimulated cells, Nrf2 is suppressed by kelch-like ECH-associated protein 1 (Keap1) which targets it for ubiquitination and proteasomal processing. Nrf2 can be activated by shear stress, dietary antioxidants (eg, sulforaphane) and other physiological stimuli which disrupt Keap1-Nrf2 interactions leading to stabilization and nuclear translocation of Nrf2.16–22 A previous study of cultured ECs revealed that activated Nrf2 can induce numerous antioxidant-defense
genes (eg, heme oxygenase-1 [HO-1]) and suppress adhesion molecule expression in ECs by inhibiting phosphorylation/activation of p38.\textsuperscript{20} However, the effects of Nrf2 on EC activation have not previously been assessed in vivo.

**Materials and Methods**

Full details of all methods can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

**In Vivo Studies**

Male C57BL/6 or Nrf2\textsuperscript{−/−} (C57BL/6)\textsuperscript{23} mice were treated with sulforaphane (5 mg/kg) 24 hours and 4 hours before experimentation. Mice were treated with LPS (4 mg/kg) for 1 hour or 6 hours before assessment of p38 phosphorylation or VCAM-1 expression, respectively. Expression levels of specific proteins were assessed in ECs at regions of the inner curvature (susceptible site) and outer curvature (protected site) of aortae by en face staining as described.\textsuperscript{9,10}

**Cell Culture Studies**

Human umbilical vein endothelial cells (HUVECs) were collected and cultured as described previously.\textsuperscript{14} Confuent cultures were exposed to unidirectional laminar shear (12 dynes/cm\textsuperscript{2}) for 24 hours using a parallel-plate flow chamber as described previously\textsuperscript{14} or were treated with sulforaphane (1 \textmu m/L). Gene-specific small interfering (si)RNAs were used to silence Nrf2, MAP kinase phosphatase-1 (MKP-1), or HO-1. Transcript levels were quantified by real-time PCR using gene-specific primers (supplemental Table) as described.\textsuperscript{9,10} MKP-1 redox state was assessed by nonreducing SDS-PAGE using total cell lysates, as described.\textsuperscript{24} The expression and intracellular localization of Nrf2 was assessed by immunostaining using anti-Nrf2 antibodies and Alexafluor 568-conjugated secondary antibodies followed by confocal microscopy. Intracellular reactive oxygen species (ROS) were measured in HUVECs loaded with the redox-sensitive fluorescent probe 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA) by quantification of fluorescence by confocal microscopy.

**Results**

**High Shear Stress Suppressed EC Activation at an Atheroprotected Region by Inducing Nrf2**

We examined whether the known spatial distribution of shear stress in the murine aorta\textsuperscript{4} correlated with Nrf2 activation in ECs. Nrf2 was localized predominantly in the nucleus of ECs in the outer curvature which is exposed to high shear stress and is protected from inflammation and atherosclerosis, but was localized predominantly in the cytoplasm of ECs in the inner curvature which is exposed to low shear and is atherosusceptible (supplemental Figure IA). Staining was not observed in parallel experiments using Nrf2\textsuperscript{−/−} animals, indicating that staining was specific for Nrf2. We confirmed that nuclear Nrf2 was active in the protected region because HO-1 (an Nrf2-target molecule) was expressed by ECs in the protected region in wild-type mice but not in Nrf2\textsuperscript{−/−} mice (supplemental Figure IB). HO-1 was not detected in the susceptible region of wild-type mice, suggesting that cytoplasmic Nrf2 is inactive at this site.

We determined whether Nrf2 regulates p38 signaling to VCAM-1 in protected regions of the aorta by performing en face staining using antibodies that recognize active phosphorylated p38 or VCAM-1. In wild-type mice, phosphorylated p38 and VCAM-1 were expressed at significantly lower levels in the protected site compared to the susceptible site in both untreated and LPS-treated mice (supplemental Figures II and III). The suppression of phosphorylated p38 in the protected region was not caused by reduced protein expression of p38 which was similar in susceptible and protected sites (supplemental Figure IV). We used Nrf2\textsuperscript{−/−} mice to examine whether Nrf2 is necessary for suppression of p38 activation and VCAM-1 expression in the protected region. Genetic deletion of Nrf2 significantly enhanced p38 phosphorylation and VCAM-1 expression at the protected site in both untreated and LPS-treated animals (supplemental Figures II and III), indicating that Nrf2 suppresses EC activation at the protected region. In contrast, genetic deletion of Nrf2 had little or no effect on EC activation at the susceptible site, which is consistent with our finding that Nrf2 is inactive at this region in wild-type mice. Interestingly, levels of phosphorylated p38 and VCAM-1 were higher in the susceptible region compared to the protected region in Nrf2\textsuperscript{−/−} mice suggesting that other negative regulators cooperate with Nrf2 to suppress EC activation at the protected site.

It is likely that Nrf2 suppresses p38–VCAM-1 signaling at the protected site as a consequence of shear stress because parallel studies performed under well defined conditions in vitro indicated that high shear stress (12 dynes/cm\textsuperscript{2} for 24 hours) significantly enhanced expression and nuclear localization of Nrf2 and simultaneously reduced expression of VCAM-1 in HUVECs (supplemental Figure VA through VD). Inhibition of Nrf2 activity using an adenosine containing a dominant negative version (Ad-Nrf2-DN) restored VCAM-1 expression in sheared HUVECs (supplemental Figure VD) indicating that endogenous Nrf2 is essential for suppression of EC activation by shear stress. Taken together, our in vitro and in vivo studies suggest that Nrf2 activation by shear stress suppresses p38 activation and VCAM-1 expression at protected sites, which may be a key molecular mechanism that governs the spatial distribution of atherosclerotic plaques.

**Sulforaphane Inhibits p38–VCAM-1 Signaling via Activation of Nrf2**

We reasoned that sites exposed to low shear may be susceptible to endothelial activation and vascular inflammation because of the absence of Nrf2 activation in ECs. Therefore, strategies to activate Nrf2 in susceptible sites may suppress vascular inflammation. To address this hypothesis we used sulforaphane, an isothiocyanate derived from cruciferous vegetables (eg, broccoli) that can activate Nrf2 by dissociating it from Keap1 and can induce antioxidants in cultured human ECs\textsuperscript{21} and in murine tissues in vivo.\textsuperscript{22} We observed that sulforaphane treatment of HUVECs did not influence Nrf2 mRNA expression (supplemental Table) but significantly enhanced expression and nuclear localization of Nrf2 protein (Figure 1A and
1B) and simultaneously reduced the expression of VCAM-1 at both the mRNA (Figure 1C and 1D) and protein levels (Figure 1E). We concluded that endogenous Nrf2 is essential for suppression of VCAM-1 expression by sulforaphane because VCAM-1 expression in sulforaphane-treated cells was restored by treatment with Ad-Nrf2-DN (Figure 1C), and by gene silencing using Nrf2-specific siRNA (Figure 1D and 1E). Pulse-chase experiments revealed that sulforaphane did not influence the stability of VCAM-1 mRNA in activated HUVECs (supplemental Figure VI), suggesting that sulforaphane inhibits VCAM-1 at a transcriptional level. To further study the molecular mechanism underlying VCAM-1 suppression by Nrf2 we examined whether sulforaphane regulates p38 or NF-κB which are essential for VCAM-1 expression. We observed that pretreatment of ECs with sulforaphane suppressed activation of p38 and MKK3/6 in response to TNFα (Figure 1F and 1G) but did not influence NF-κB activation (Figure 1G). Ad-Nrf2-DN restored p38 activation in sulforaphane-treated HUVECs (Figure 1F), indicating that sulforaphane inhibits p38 activity via Nrf2. Similarly, overexpression of Nrf2 in HUVECs (supplemental Figure VII) using an adenovirus (A; Ad-Nrf2) suppressed induction of VCAM-1 (B) and activation of p38 and MKK3/6 (C and D) by TNFα but did not influence NF-κB (E).

Given that sulforaphane induces antioxidants, we hypothesized that it suppresses p38 activation by altering cellular ROS. Consistent with this idea, treatment of HUVECs with sulforaphane (Figure 1H) or Ad-Nrf2 (supplemental Figure VIII) suppressed the induction of cellular ROS. Moreover, screening of a panel of antioxidants by quantitative PCR revealed that sulforaphane induces HO-1, aldo-keto reductase family 1 members C1 and C3,
and thioredoxin reductase 1 (supplemental Table). As the induction of HO-1 was most prominent, we examined its role in EC responses to sulforaphane. Inhibition of HO-1 activity using zinc protoporphyrin or silencing of HO-1 expression using prevalidated siRNA sequences did not influence VCAM-1 suppression by sulforaphane (supplemental Figure VIII), suggesting that HO-1 is not essential for the antiinflammatory effects of sulforaphane. It is plausible that the other Nrf2-induced antioxidants can compensate for the absence of HO-1 activity in sulforaphane-treated cells. We next examined whether sulforaphane suppresses p38 activation via MKP-1, an antiinflammatory negative regulator of p38 that is expressed by ECs in atheroprotected regions. Sulforaphane did not induce MKP-1 expression (supplemental Table). However, silencing of MKP-1 significantly elevated VCAM-1 expression in sulforaphane-treated HUVECs (Figure 1I), indicating that endogenous MKP-1 is involved in suppression of VCAM-1 expression by sulforaphane. A previous study demonstrated that ROS can inactivate MKP-1 by oxidizing catalytic cysteine residues to sulfenic acid, thus promoting the formation of disulfide bonds between MKP-1 and cellular proteins. Given that Nrf2 reduces ROS, we examined whether Nrf2 can enhance MKP-1 activity by promoting the reduced form. Analysis of cell lysates by SDS-PAGE in the absence of reducing agents revealed both oxidized and reduced forms of MKP-1, which displayed a marked difference in electrophoretic mobility (supplemental Figure VIIG), as described previously. Treatment of HUVECs with H2O2 induced a high-molecular-weight disulfide-linked form of MKP-1 (oxidized; compare 1 and 2) that could be reduced by incubation of cell lysates with DTT (compare 2 and 4). The reduced form of MKP-1 was promoted by overexpression of Nrf2 using Ad-Nrf2 (compare 2 and 3), suggesting that Nrf2 can enhance MKP-1 catalytic activity by influencing its redox state.

Pharmacological Activation of Nrf2 Suppresses EC Activation in a Susceptible Region of the Murine Aorta

We examined whether pharmacological activation of Nrf2 using sulforaphane can reduce proinflammatory activation at atherosusceptible sites. Treatment of mice with sulforaphane activated Nrf2 in a susceptible region of the aorta (Figure 2). Sulforaphane treatment also reduced p38 activation and VCAM-1 expression at the susceptible site in wild-type mice (Figures 3 and 4, compare 5 with 7) but had no effect in Nrf2/H2O2 mice (compare 6 with 8), indicating that sulforaphane suppresses p38–VCAM-1 signaling at the susceptible site by activating Nrf2. At the protected region of wild-type animals, Nrf2 was constitutively active and sulforaphane treatment had relatively modest effects on Nrf2 activity (Figure 2), p38 phosphorylation (Figure 3), and VCAM-1 expression (Figure 4).

Discussion

Studies using cultured ECs suggest that the molecular mechanism underlying the antiinflammatory effects of shear stress involves the transcription factors KLF2 and Nrf2, both of which can be activated by shear stress and can limit proinflammatory activation when overexpressed in ECs. Indeed KLF2 and Nrf2 can act synergistically to modulate the transcriptome in cultured ECs exposed to shear stress. The involvement of KLF2 has been confirmed by studies of human, murine and zebrafish arteries which have demonstrated that KLF2 is expressed at high shear regions. In contrast, the regulation and physiological role of Nrf2 in the vasculature in vivo has thus far received little attention. Although previous studies have revealed that Nrf2 is expressed in the descending thoracic aorta and can be activated by oxidized phospholipids in the carotid artery, the capacity of Nrf2 to regulate proinflammatory activation of ECs in arteries has not been previously investigated. Here we demonstrate that Nrf2 is constitutively active in ECs at a high-shear atheroprotected region of the murine aorta and that it reduces proinflammatory activation at this site by inactivating p38 MAP kinase and suppressing VCAM-1 expression. Thus Nrf2 activation by shear stress may be a key molecular mechanism that governs the spatial distribution of atherosclerotic plaques. Interestingly, we observed that genetic deletion of Nrf2 did not entirely restore...
proinflammatory activation at the protected site, suggesting that Nrf2 acts in concert with other antiinflammatory molecules (eg, KLF2,26–28) to inhibit proinflammatory activation in vascular endothelium exposed to high shear. We observed that ECs at the atherosusceptible site expressed an inactive form of Nrf2 which was localized to the cytoplasm and was incapable of suppressing proinflammatory activation, and concluded that regions exposed to low or oscillatory shear may be susceptible to inflammation attributable to the absence of Nrf2 activation in ECs. The corollary is that drugs that can activate Nrf2 independently of shear stress may be able to suppress inflammation and atherosclerosis at susceptible sites. To test this concept we studied sulforaphane, a dietary antioxidant that acts as a potent activator of Nrf2.21,22 Our studies of cultured ECs revealed that a relatively low concentration of sulforaphane (1 μmol/L) elevated Nrf2 expression at the protein level but did not enhance Nrf2 transcript levels, a
finding that is consistent with the observation that sulforaphane can stabilize Nrf2 by inhibiting Keap1.32 We observed that sulforaphane suppressed p38 activation and VCAM-1 expression in cultured ECs and subsequent studies, in which Nrf2 was either silenced or inactivated, revealed that Nrf2 was required for these anti-inflammatory effects. These data are consistent with our observation that sulforaphane suppressed endothelial activation at a susceptible site in wild-type mice but not in Nrf2−/− animals. A recent study demonstrated that relatively high concentrations of sulforaphane can influence EC activation through Nrf2-independent mechanisms,33 and we therefore conclude that sulforaphane may have concentration-dependent effects on proinflammatory signaling pathways.

Our study demonstrates that Nrf2 suppresses endothelial activation by targeting the MAP kinase signaling pathway at 2 levels, ie, by reducing MKK3/6 signaling to p38 and by enhancing the activity of MKP-1, an anti-inflammatory molecule that inactivates p38 by dephosphorylation. Nrf2 did not regulate MKP-1 at the level of expression. Instead, we provide evidence that Nrf2 can enhance the catalytic activity of MKP-1 by promoting a reducing environment via the induction of multiple antioxidants. We have previously demonstrated that MKP-1 is induced by shear stress and is preferentially expressed by ECs in a protected region of the murine aorta where it inhibits p38–VCAM-1 signaling.10 Thus we propose that shear stress suppresses EC activation at atheroprotected sites by inducing MKP-1 and by simultaneously enhancing MKP-1 activity via activation of Nrf2. Our studies revealed that Nrf2 also suppresses the activation of MKK3/6, a signaling intermediary that activates p38 by phosphorylation. The underlying mechanism for MKK3/6 suppression by Nrf2 may involve redox regulation as well because ASK1, a MAP kinase kinase kinase that acts upstream from MKK3/6, is known to be inhibited by reduced forms of glutathione and thioredoxin.34,35

We believe that sulforaphane is the first example of a therapeutic intervention that enhances the activity of an anti-inflammatory transcription factor at atherosusceptible regions, and that this compound may have clinical utility for the prevention or treatment of vascular inflammation. In addition, given that cruciferous vegetables are a source of sulforaphane it is plausible that their known beneficial effects on cardiovascular health66 involve activation of Nrf2 at atherosusceptible regions. We administered sulforaphane to mice i.p. because dosing this route can be controlled more accurately than oral administration. The dose used in our study was based on the efficacy of sulforaphane established in previous studies.37,38 Further animal studies are now required to assess directly whether the consumption of green vegetables can influence Nrf2 activation and inflammation at susceptible sites. A recent study revealed that genetic deletion of Nrf2 reduces atherosclerosis in ApoE−/− mice, and demonstrated that Nrf2 exerts proatherogenic effects in cultured macrophages by positively regulating the expression of CD36 which is a scavenger receptor for modified LDL.39 However, our current findings indicate that Nrf2 may exert antiatherogenic effects in vascular endothelium by suppressing inflammation. Thus, activation of Nrf2 in endothelial cells and macrophages may exert opposing effects on lesion development. Studies using conditional knockouts of Nrf2 are now required to assess the functions of Nrf2 in specific vascular cells in relation to atherosclerosis.

Sources of Funding
The study was funded by the British Heart Foundation.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2009;29:1851-1857; originally published online September 3, 2009;
doi: 10.1161/ATVBAHA.109.193375
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Animals Male C57BL/6 mice between 2 and 3 months of age were used. The Nrf2 knockout mouse strain (Nrf2−/− (C57BL/6))1 was generously supplied by Prof. Masayuki Yamamoto, University of Tsukuba, Japan via the RIKEN BioResource Center, Tsukuba, Japan. Mice were treated with sulforaphane (5 mg/kg in 10% corn-oil/ 90% PBS) by intraperitoneal injections 24h and 4h prior to experimentation. Mice were treated with LPS (4mg/kg) by intraperitoneal injection for 1 hour or 6 hours prior to assessment of p38 phosphorylation or VCAM-1 expression, respectively. All experiments were performed within guidelines set out by the Federation of European Laboratory Animal Science Associations.

Reagents and antibodies Human recombinant TNFα and LPS (R&D) and anti-phosphorylated-MKK3/6 (Cell signalling Technology), anti-phosphorylated-p38 Tyr180/Thr182 (Cell signalling Technology), anti-p38 (Cell Signaling Technology), anti-RelA (Santa Cruz), anti-CD31-FITC (BD Biosciences Pharmingen), anti-Nrf2 (Santa Cruz Biotechnology), anti-HO-1 (Santa Cruz Biotechnology), anti-tubulin (Sigma Aldrich) and anti-lamin B (Affinity BioReagents) antibodies were obtained commercially. TNFα was used at a final concentration of 10ng/ml. The generation of anti-VCAM-1 (1.G11) antibodies was described previously2. Anti-MKK3/6 antibodies3 were generously supplied by Dr Jonathan Dean, Imperial College London, UK. Adenoviruses containing wild-type Nrf2 (Ad-Nrf2) or a dominant negative version that lacks the transactivation domain (Ad-Nrf2-DN) were generously provided by Prof. Jeffrey A. Johnson, University of Wisconsin-Madison, USA and have been described
previously\textsuperscript{4}. Other reagents were purchased from Sigma Aldrich unless otherwise stated.

\textbf{En face staining} The expression levels of specific proteins were assessed in EC at regions of the inner curvature (susceptible site) and outer curvature (protected site) of murine aortae by \textit{en face} staining as described previously\textsuperscript{5,6}. Animals were sacrificed by CO\textsubscript{2} inhalation. Aortae were perfused \textit{in situ} with PBS (at a pressure of approximately 100mm Hg) and then perfusion-fixed with 2\% formalin prior to harvesting. Fixed aortae were tested by immunostaining using specific primary antibodies and Alexafluor568-conjugated secondary antibodies (red). EC were identified by co-staining using anti-CD31 antibodies conjugated to the fluorophore FITC (green). Nuclei were identified using a DNA-binding probe with far-red emission (To-Pro-3; Invitrogen). Stained vessels were mounted prior to visualization of endothelial surfaces \textit{en face} using confocal laser-scanning microscopy (Zeiss LSM 510 META). Isotype-matched monoclonal antibodies raised against irrelevant antigens or pre-immune rabbit sera were used as experimental controls for specific staining (data not shown). The expression of particular proteins at each site was assessed by quantification of fluorescence intensity for multiple cells (at least 100 per site) using LSM 510 software (Zeiss) and calculation of mean fluorescence intensities with standard deviations.

\textbf{Endothelial cell culture} Human umbilical vein endothelial cells (HUVEC) were collected using collagenase and cultured as described previously\textsuperscript{7}. Confluent HUVEC cultures were exposed to high unidirectional laminar shear (12 dynes/cm\textsuperscript{2}) for 24h using a parallel-plate flow chamber (Cytodyne) as described previously\textsuperscript{7}. A stock solution of sulforaphane (1 mM in DMSO) was used to treat HUVEC to achieve a final
concentration of 1 μM sulforaphane. Alternatively, HUVEC were treated with an equivalent volume of DMSO alone as a control.

**Gene silencing.** RNA interference was carried out using small interfering (si)RNAs that specifically target Nrf2 (5'-AAGGATTATTATGACTGTTAA-3'; Qiagen), MAP kinase phosphatase-1 (MKP-1) (5'-AAGCUGGACGAGGCUUUGAGUU-3'; Dharmacon) or HO-1 (5'-UGCUGAGUUCAUGAGGAACUU-3'; Dharmacon). Alternatively, cells were treated with non-targeting scrambled controls (Silencer Negative Control no. 1 siRNA; Ambion, Foster City, Calif, or non-targeting siRNA no.1; Thermo Fisher Scientific). Cell cultures that were 80% to 90% confluent were transfected with siRNA (5 μM final concentration) by microporation (Digital BioTechnology, Seoul, Korea) following the manufacturer’s instructions and then incubated in growth medium without antibiotics for 48h before analysis.

**Comparative real time PCR** Transcript levels were quantified by comparative real-time PCR using gene-specific primers (Supplementary Table). Extraction and reverse transcription of total RNA and real-time PCR were carried out as described previously. Reactions were performed in triplicate. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product as described previously.

**Flow cytometry** HUVEC were harvested and incubated on ice for 1 hour with mouse IgG antibodies specific for VCAM-1. After washing and application of fluorescein isothiocyanate-conjugated secondary antibody, cells were analysed by flow cytometry after gating out dead cells. The relative fluorescence index (RFI) was calculated after
subtracting background fluorescence measured using secondary-only controls.

**Western blotting** Cytosolic or nuclear lysates were prepared using the Nuclear Extraction Kit (Active Motif). MKP-1 redox state was assessed by non-reducing SDS-PAGE using total cell lysates, as described. Western blotting was carried out using specific primary antibodies, horse radish peroxidase-conjugated secondary antibodies and chemiluminescent detection.

**Assay of NF-κB DNA binding** Binding of RelA NF-κB sub-units to consensus oligonucleotides was assessed by DNA-binding ELISA (Active Motif) using nuclear lysates prepared using the NucBuster kit (Novagen).

**Immunofluorescent staining of Nrf2 in HUVEC** The expression and intracellular localization of Nrf2 in cultured HUVEC was assessed by immunostaining of methanol-fixed cells using anti-Nrf2 antibodies and Alexafluor 568-conjugated secondary antibodies followed by laser-scanning confocal microscopy (LSM 510 META; Zeiss). Nuclei were identified using the DNA-binding probe To-Pro-3. Image analysis was performed using Zeiss LSM 510 META software to calculate average fluorescence values after subtracting background fluorescence values from cells stained with secondary antibody alone.

**Measurement of intracellular reactive oxygen species (ROS).** Intracellular ROS were measured in HUVEC cultured on glass slide chambers. They were incubated with the redox-sensitive fluorescent probe 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA, 10 μM; Invitrogen) for 45 minutes and then washed with
phosphate-buffered saline prior to experimentation. Fluorescence was quantified in live cells using a laser-scanning confocal microscope with a specialized stage that maintained cultures at 37°C in a 5% CO2 environment (LSM 510 META; Zeiss).

Statistics Differences between samples were analysed using an unpaired Student’s t-test or one-way ANOVA with Bonferroni adjustment (*p<0.05, **p<0.01, ***p<0.001).
REFERENCES FOR SUPPLEMENT MATERIAL


LEGENDS FOR SUPPLEMENTARY TABLE AND FIGURES

Supplementary Table

**Effects of sulforaphane on gene expression.** HUVEC were treated with SFN (1 μM) or with vehicle alone and incubated for 4 hours. Levels of particular transcripts were quantified by real-time PCR using gene-specific primers (sequences indicated) and were normalised by measuring β-actin transcript levels. Mean values (+/- SD) calculated from two independent experiments are shown.

**Supplementary Fig. I**

**Nrf2 is activated in EC at a protected site in the aorta.** Nrf2 expression levels and intracellular localization (A) or HO-1 expression levels (B) were assessed in EC at protected (P) or susceptible (S) regions of the aorta in wild-type or Nrf2+/− mice by *en face* staining using anti-Nrf2 or anti-HO-1 antibodies. Endothelial marker (CD31); nuclear counterstain (DNA). Representative images and quantitation of nuclear Nrf2 (A) and HO-1 (B) from multiple animals are shown.

**Supplementary Fig. II**

**Nrf2 suppresses p38 activation in EC in a protected region of the aorta.** Phosphorylated p38 (phos-p38) levels were assessed by *en face* staining of protected or susceptible regions in wild-type or Nrf2+/− mice (treated with LPS or untreated; n=4 per group). Endothelial marker (CD31); nuclear counterstain (DNA). Representative images and quantitation of p38 phosphorylation from multiple animals are shown.
Supplementary Fig. III

**Nrf2 suppresses VCAM-1 expression in EC in a protected region of the aorta.**
VCAM-1 expression levels were assessed by *en face* staining of protected or susceptible regions in wild-type or Nrf2°/° mice (treated with LPS or untreated; n=4 per group). Endothelial marker (CD31); nuclear counterstain (DNA). Representative images and quantitation of VCAM-1 expression from multiple animals are shown.

Supplementary Fig. IV

**Expression levels of p38 are similar in protected and susceptible regions of wild-type and Nrf2°/° mice.** p38 expression levels were assessed by *en face* staining of protected or susceptible regions in wild-type or Nrf2°/° mice. Endothelial marker (CD31); nuclear counterstain (DNA). Representative images and quantitation of p38 expression from multiple animals are shown.

Supplementary Fig. V

**Nrf2 is essential for suppression of pro-inflammatory activation by shear stress in cultured EC.** HUVEC were exposed to unidirectional shear stress for 24 hours or were cultured under static conditions. (A) Levels of Nrf2 transcripts were quantified by real-time PCR. Mean values (+/- SD) calculated from three independent experiments are shown. (B) Cytosolic or nuclear lysates were tested by Western blotting using anti-Nrf2, anti-tubulin or anti-lamin B antibodies. Representative of three independent experiments. (C) Nrf2 expression and intra-cellular localization was assessed by immunofluorescence staining using anti-Nrf2 antibodies and nuclear counterstaining (DNA). Representative images and mean nuclear Nrf2 levels (+/- SD) calculated from multiple EC are shown. (D) HUVEC were transduced with Ad-Nrf2-DN or with an
empty adenovirus (Ad-empty) or remained untreated and were incubated for 24 hours. Cells were then either stimulated with TNFα for 4 hours or remained untreated. Levels of VCAM-1 transcripts were quantified by real-time PCR. Mean values (+/- SD) calculated from three independent experiments are shown.

**Supplementary Fig. VI**

**Sulforaphane does not influence VCAM-1 mRNA stability**

The effect of sulforaphane (SFN) on the stability of VCAM-1 mRNA was assessed by pulse-chase experiments. HUVEC were stimulated with TNFα for 4 hours or remained untreated. Activated cells were subsequently incubated with actinomycinD (50 μM) to block transcription, either in the presence of SFN (1 μM) or vehicle alone. VCAM-1 transcript levels were then measured at various time points by real-time PCR. Mean values (+/- SD) calculated from two independent experiments are shown.

**Supplementary Fig. VII**

**Nrf2 suppresses p38-VCAM-1 signalling**

(A) The effect of an Nrf2-containing adenovirus (Ad-Nrf2) on HO-1 expression levels was determined. HUVEC were transduced with Ad-Nrf2 or with an empty adenovirus (Ad-empty) and incubated for 24 hours. Cytosolic lysates were tested by Western blotting using anti-Nrf2, anti-HO-1 or anti-tubulin antibodies. Representative of three independent experiments. (B, C, D, E, F) The effect of Nrf2 overexpression on pro-inflammatory signalling and on the induction of ROS was determined. HUVEC were transduced with Ad-Nrf2, Ad-Nrf2-DN or Ad-empty and were incubated for 24 hours, or were untreated. Cells were then stimulated with TNFα for 30 minutes (D), 4 hours (B) or varying times (C, E). (B) Levels of VCAM-1 transcripts were quantified by real-
time PCR. Mean values (+/- SD) calculated from triplicate measurements are shown. Representative of three independent experiments. (C, D) Cell lysates were tested by Western blotting using antibodies that recognise phosphorylated p38 (phos-p38), total p38, phosphorylated MKK3/6 or total MKK3/6. Representative of three independent experiments. (E) NF-κB activation was assessed by DNA-binding ELISA. Mean optical densities (450 nm) calculated from triplicate wells are shown with standard deviations. Representative of three independent experiments. (F) HUVEC were loaded with the redox-sensitive probe H$_2$DCFDA, treated with H$_2$O$_2$ (50 μM) and fluorescence was assessed at varying times by confocal microscopy. Representative images and quantitation of fluorescent cells after 5 minutes stimulation (mean +/- SD; pooled from 3 experiments) are shown. (G) The effect of Nrf2 expression on MKP-1 redox state was determined. Cells were treated with 0.3 μM H$_2$O$_2$ for 10 minutes or remained untreated as a control. Total cell lysates were analysed by SDS-PAGE in the presence or absence of DTT (as indicated) followed by Western blotting using anti-MKP-1 antibodies. Oxidised and reduced forms of MKP-1 are indicated. NS, non-specific band.

**Supplementary Fig. VIII**

**HO-1 activity is not required for suppression of VCAM-1 expression by sulforaphane.** The role of HO-1 in EC responses to SFN was assessed. (A) HUVEC were treated with zinc protoporphyrin (ZnPP; 10 μM for 24 hours) which is a pharmacological inhibitor of HO-1 or with vehicle alone. (B) Alternatively, HUVEC were transfected with HO-1 specific siRNA or with a scrambled control (Scr). (A, B) Cells were then exposed to SFN (1 μM) or vehicle alone for 4 hours, and then stimulated with TNFα for 4 hours or remained untreated. Levels of HO-1 or VCAM-1
transcripts were quantified by real-time PCR. Mean values (+/- SD) calculated from triplicate measurements are shown.
## SUPPLEMENTARY TABLE

<table>
<thead>
<tr>
<th>SFN</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1 *</td>
<td>1.48 ± 0.71</td>
<td>6.60 ± 1.56 5'-CGAAACAGCAGAACCAGTCT-3' 5'-AGCCCTTCTGAGCT-3'</td>
</tr>
<tr>
<td>Aldo-keto reductase fam 1 C3 ***</td>
<td>1.00 ± 0.00</td>
<td>2.69 ± 0.07 5'-AATTAACATAAGCTTGTT-3' 5'-AGTGCTAAACAGGAGAT-3'</td>
</tr>
<tr>
<td>Aldo-keto reductase fam 1 C1 *</td>
<td>1.19 ± 0.28</td>
<td>2.45 ± 0.07 5'-AAACAGCCAGGCTAAAGT-3' 5'-GATTTGAGAAATCCGAG-3'</td>
</tr>
<tr>
<td>Thioredoxin reductase 1 *</td>
<td>1.08 ± 0.14</td>
<td>1.79 ± 0.14 5'-CCACTTGGTGAAGACCGTT-3' 5'-AGGAGAAAGATCATACTCTGAT-3'</td>
</tr>
<tr>
<td>Aldo-keto reductase fam 1 C2</td>
<td>1.34 ± 0.50</td>
<td>1.99 ± 0.42 5'-GCTTGATTTCTGCAAAGTCAA-3' 5'-GTCCACCCATTGGTTTCTTC-3'</td>
</tr>
<tr>
<td>UDP-Glucose dehydrogenase</td>
<td>1.13 ± 0.21</td>
<td>1.42 ± 0.35 5'-CATCCAGGTTTTACAGGA-3' 5'-GCAAATAACACAGCCTTGG-3'</td>
</tr>
<tr>
<td>Serum- and glucose-regulated kinase 1</td>
<td>1.14 ± 0.21</td>
<td>1.39 ± 0.00 5'-GCTCGTCTTCTATGCTGTA-3' 5'-AGGAAATGTTCTGGTGTA-3'</td>
</tr>
<tr>
<td>Ferritin light chain</td>
<td>1.05 ± 0.07</td>
<td>1.38 ± 0.35 5'-GCCGAGGGCTCTAGGAG-3' 5'-CTATGGCTGGAGGGAG-3'</td>
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<tr>
<td>γ-glutamylcysteine synthetase heavy chain</td>
<td>1.14 ± 0.21</td>
<td>1.79 ± 0.78 5'-TGAGGCCAACATGGCAAAAC-3' 5'-AAATCACCTCCAAGCAATC-3'</td>
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<tr>
<td>Thioredoxin</td>
<td>1.19 ± 0.28</td>
<td>1.23 ± 0.35 5'-AGGGAACAAAAGGATGATGAT-3' 5'-AGTTTAAATAGCCAATGCT-3'</td>
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<tr>
<td>Microsomal epoxide hydrolase</td>
<td>1.09 ± 0.14</td>
<td>1.09 ± 0.14 5'-TGCGGCGCCGCTCTCTCAGA-3' 5'-CAGCGACGCCCACCTCCCTCA-3'</td>
</tr>
<tr>
<td>NAD(P)H:quinone oxidoreductase 1</td>
<td>0.88 ± 0.14</td>
<td>1.07 ± 0.28 5'-TCAATTCTCCTGGCACATCAGAAG-3' 5'-GGAGTGTCCTGACAGC-3'</td>
</tr>
<tr>
<td>Ferritin heavy chain</td>
<td>0.91 ± 0.14</td>
<td>1.08 ± 0.07 5'-CGACCCGCTCCACCTCG-3' 5'-CTTCATTATATCTCGTCC-3'</td>
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<tr>
<td>Manganese superoxide dismutase</td>
<td>1.07 ± 0.07</td>
<td>1.00 ± 0.42 5'-CGACCTGCGGTACGACTAT-3' 5'-TTCAGGTTCCAGGTAGGC-3'</td>
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<tr>
<td>Glutathione S-transferase P1-1</td>
<td>0.92 ± 0.14</td>
<td>0.82 ± 0.14 5'-GCTTTATGGGAAGGGACAG-3' 5'-CTCAAAGGCGCTGTTTGC-3'</td>
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<tr>
<td>Nrf2</td>
<td>1.00 ± 0.00</td>
<td>0.86 ± 0.13 5'-TACTCCCGAAGTCCGACCACA-3' 5'-CATCTCAACAGGGAATACT-3'</td>
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<tr>
<td>MKP-1</td>
<td>1.00 ± 0.00</td>
<td>0.66 ± 0.14 5'-CAGCTGCTGAGTCTGAGTC-3' 5'-AGGTAAGTCAGCGCAGTGT-3'</td>
</tr>
</tbody>
</table>
Supplementary Fig. I

A  
Susceptible (S)  |  Protected (P)  |  Protected (P)  
CD31  |  Nrf2  |  CD31  |  Nrf2  |  CD31  |  Nrf2  
DNA  |  merge  |  DNA  |  merge  |  DNA  |  merge  
Wild-type  |  |  |  Nrf2^{-/-}  

Nuclear Nrf2

Fluorescence intensity

***  
Wild-type  |  Nrf2^{-/-}  

B  
Susceptible (S)  |  Protected (P)  |  Protected (P)  
CD31  |  HO-1  |  CD31  |  HO-1  |  CD31  |  HO-1  
DNA  |  merge  |  DNA  |  merge  |  DNA  |  merge  
Wild-type  |  |  |  Nrf2^{-/-}  

Fluorescence intensity

***  
Wild-type  |  Nrf2^{-/-}
Supplementary Fig. II

Fluorescence intensity

LPS

Protected

Susceptible

Wild-type

Nrf2 -/-

+ LPS

untreated

Fluorescence intensity
Supplementary Fig. III

![Image of fluorescence intensity graph and microscopy images showing CD31 and VCAM-1 expression in Wild-type and Nrf2⁻/⁻ mouse models under untreated and LPS-treated conditions. The graph compares fluorescence intensity between Protected and Susceptible groups. The images show marked differences in fluorescence intensity and pattern of CD31 and VCAM-1 expression in response to LPS treatment.]
Supplementary Fig. IV
Supplementary Fig. V

**A**

![Graph showing Nrf2 mRNA levels](image)

**B**

![Western blots showing Nrf2, tubulin, and lamin B](image)

**C**

![Immunofluorescence images showing Nrf2, DNA, and merge](image)

**D**

![Bar graph showing VCAM-1 mRNA levels](image)
Supplementary Fig. VI

![Graph showing VCAM-1 mRNA levels over time with DMSO and SFN treatments](image)
Supplementary Fig. VII

A

Nrf2
HO-1
Tubulin

MOI 500 100 200 400 500
Ad-empty Ad-Nrf2

B

VCAM-1 mRNA levels

Ad-Ad Ad-Nrf2

p38
phos-p38

10 15 30 60 90 0 10 15 30 60 90 0 10 15 30 60 90 0 10 15 30 60 90

C

phos-p38
p38
TNFα (min)

No adenovirus Ad-empty Ad-Nrf2 Ad-Nrf2-DN

D

phos-MKK3/6
MKK3/6

TNFα - + + +
Ad-empty + + + +
Ad-Nrf2 - - + -

E

NF-κB DNA binding

Ad-empty Ad-Nrf2 Ad-Nrf2

F

Ad-empty Ad-Nrf2

Time (min) 0 15 15 30 30

G

97 kD 64 kD 51 kD 39 kD
H2O2 DTT

oxidised reduced ns ns ns

Ad-empty + + - +
Ad-Nrf2 - - + -

DTT 1 2 3 4
Supplementary Fig. VIII

**A**

![Graph showing VCAM-1 mRNA levels with different treatments](image)

**B**

![Graph showing VCAM-1 and HO-1 mRNA levels with different treatments](image)
Fig. 2

Susceptible vs. Protected

Vehicle

SFN

Nuclear Nrf2

Fluorescence intensity

SFN

- +

susceptible protected

***
**Fig. 3**

- **Wild-type**
  - CD31 phos-p38
  - DNA merge
  - SFN

- **Nrf2-/-**
  - CD31 phos-p38
  - DNA merge
  - SFN

Fluorescence intensity:
- 0
- 50
- 100
- 150
- 200
- 250

- ---++ - -++
- Protected Susceptible

**Graph**
- Wild type
- Nrf2-/-
- SFN

<table>
<thead>
<tr>
<th>SFN</th>
<th>Protected</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

---

**Legend**
- Wild type
- Nrf2-/-

***
Fig. 4

Fluorescence intensity

---

**Wild-type**

1. CD31, VCAM-1
2. DNA, merge

**Nrf2**

3. CD31, VCAM-1
4. DNA, merge

---

**Protected**

5. CD31, VCAM-1
6. DNA, merge

---

**Susceptible**

7. CD31, VCAM-1
8. DNA, merge

---

Fluorescence intensity

---

**Wild type**

- 1: SFN -
- 2: SFN +
- 3: SFN -
- 4: SFN +

**Nrf2**

- 5: SFN -
- 6: SFN +
- 7: SFN -
- 8: SFN +

---

**Protected**

---

**Susceptible**

---

***