Nrf2 Gene Transfer Induces Antioxidant Enzymes and Suppresses Smooth Muscle Cell Growth In Vitro and Reduces Oxidative Stress in Rabbit Aorta In Vivo

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**Background**—Reactive oxygen species (ROS) play a major role in vascular inflammation and pathophysiology of many vascular diseases such as atherosclerosis and injury-induced neointima formation after balloon angioplasty. Nuclear factor E2–related factor-2 (Nrf2) is a transcription factor orchestrating antioxidant and cytoprotective responses on oxidative and electrophilic stress, and it has been shown to have antiinflammatory effects in vascular cells in vitro. We therefore postulated that Nrf2 gene transfer would have salutary effects on vascular inflammation after angioplasty.

**Methods and Results**—Transduction of vascular smooth muscle cells (VSMCs) with Nrf2-expressing adenovirus increased the expression of several antioxidant enzymes including heme oxygenase-1 (HO-1) compared with β-galactosidase (AdLacZ)-transduced controls. Moreover, Nrf2 gene transfer also inhibited vascular smooth muscle cell (VSMC) proliferation, and the effect was partially reversed by the HO inhibitor Sn(IV) protoporphyrin. In vivo, adenoviral gene transfer effectively reduced oxidative stress determined by antibody staining against oxidized epitopes of LDL, as well as inhibited vascular inflammation assessed by the macrophage cell count and monocyte chemoattractant protein-1 (MCP-1) staining. However, the antiproliferative effects of Nrf2 in vivo were counterbalanced with diminished apoptosis in neointimal VSMCs, resulting in no change in neointimal hyperplasia.

**Conclusions**—Nrf2 gene transfer or Nrf2-inducing drugs may have therapeutic applications in vascular diseases in which inflammation and oxidative stress play a role. However, the contrasting growth inhibitory and antiapoptotic effects of Nrf2 need to be considered in pathological conditions in which SMC proliferation plays a critical role. (Arterioscler Thromb Vasc Biol. 2007;27:741-747.)

**Key Words:** antioxidants ■ free radicals ■ gene therapy ■ restenosis

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**Reactive oxygen species (ROS)** play a role in a number of cardiovascular pathologies, including response to arterial injury after balloon angioplasty. ROS derived mainly from vascular smooth muscle cells (VSMCs) contribute to the proliferation and migration of medial VSMCs leading to neointimal hyperplasia and adverse remodeling and ultimately, vessel restenosis.\(^1\) Particularly, the role of NAD(P)H oxidases as a source of ROS in VSMCs leading to neointimal hyperplasia has been demonstrated.\(^2\) Also, sustained downregulation of the expression of extracellular superoxide dismutase (EC-SOD) has been shown to occur after balloon angioplasty, contributing to the imbalance between the production and disposal of ROS and constrictive remodelling.\(^3\) Importantly, therapeutic approaches aiming at augmenting the antioxidant defense, such as gene therapy with antioxidant genes heme oxygenase-1 (HO-1)\(^4\) or EC-SOD,\(^5\) inhibit injury-induced neointima formation in animal models of restenosis. Although the evidence for the role of oxidative stress in restenosis from human studies is weaker, an antioxidant probucol and its stable modification AGI-1067 have been shown to reduce restenosis after percutaneous coronary intervention (PCI).\(^6\) Interestingly, it has been recently shown in animal models of atherosclerosis and restenosis that the beneficial effects of probucol are likely not mediated via its direct antioxidant actions but through induction of HO-1,\(^7\) suggesting that the augmentation of endogenous antioxidant defense may have potential for their treatment.

Nuclear factor E2-related factor-2 (Nrf2) is a member of CNC (cap ‘n’ collar) family of b-Zip transcription factors and an indispensable positive regulator of many antioxidant and phase II detoxifying enzymes.\(^8\) On activation by oxidative or electrophilic stress, Nrf2 protein stabilizes, translocates to the nucleus, heterodimerizes with small Maf proteins, and binds to the so-called antioxidant response element (ARE), a
common regulatory element found in the 5’-flanking regions of antioxidant and detoxification enzymes. There is a large number of genes regulated by ARE, including enzymes involved in glutathione (GSH) metabolism, such as the subunits of the rate-limiting enzyme of glutathione synthesis, glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunit genes. Also NAD(P)H:quinone oxidoreductase-1 (NQO1), which not only detoxifies xenobiotic quinones, but also reduces antioxidants vitamin E and coenzyme Q₁₀ to their active form, is a Nrf2 target gene. In addition, HO-1 has been shown to be positively regulated by Nrf2.

Gene therapy with transcription factors enables concerted induction or repression of multiple target genes, which may be beneficial when aiming at integrated responses in situations requiring the interplay of several factors having a common regulatory pathway. Examples of such approaches include the use of constitutively active hypoxia inducible factor-1α (HIF-1α) for the induction of angiogenic growth factors and therapeutic revascularization, or Sonic hedgehog (Shh) gene transfer for the augmentation of multiple trophic factors and myocardial tissue regeneration. In analogy, simultaneous induction of antioxidant genes in situations in which oxidative stress contributes to the pathophysiology may be a better approach than gene transfer with individual antioxidant genes. Our goal was therefore to test a novel approach for augmenting antioxidant defenses by using concerted induction of antioxidant genes by adenoviral Nrf2 gene transfer. To this end, we first tested the efficacy of Nrf2 gene transfer in vitro to induce antioxidant genes in VSMCs, followed by studies assessing the in vivo effects of Nrf2 in the rabbit aortic balloon denudation model.

Materials and Methods
The expression of Nrf2, HO-1, GCLC, GCLM, and NQO1 in human aortic smooth muscle cells (HASMCs) transduced with Nrf2 or LacZ expressing adenovirus was studied using real time-PCR or Western blotting. The mRNA expression of HO-1 in transduced rabbit aortic smooth muscle cells (RaASMCs) was assessed by real time-PCR and the HO-1 activity spectrophotometrically. Cell proliferation was determined by [3H]-thymidine incorporation assay. The intraarterial gene transfer was performed as previously described, and the arteries were harvested for RNA and histological analyses 7 days, 14 days, and 28 days after gene transfer. For more details, please see the supplemental materials, available online at http://atvb.ahajournals.org.

Results
AdNrf2 Overexpression Induces Antioxidant Genes in HASMCs and RaASMCs
The VSMCs is the primary cell type transduced by adenovirus in the rabbit balloon denudation model, and also the major cell type contributing to neointimal hyperplasia after balloon injury. We therefore first studied the effect of Nrf2 gene transfer on antioxidant gene expression in HASMCs. The cells were transduced with Nrf2 expressing or LacZ control adenovirus, and the gene expressions of Nrf2, HO-1, GCLC, GCLM, and NQO1 were studied at the RNA and
protein levels 48 hour post-transduction using real time-PCR and Western blotting. Transduction of HASMCs with increasing MOIs of AdNrf2 effectively increased both Nrf2 mRNA and protein expression (Figure 1A and 1C). Furthermore, Nrf2 gene transfer was able to induce the known target genes of Nrf2, most notably HO-1 (Figure 1B, 1C; supplemental Figures I and II). Also AdLacZ caused a small but significant increase in the expression of HO-1 mRNA in comparison to no virus controls (Figure 1B). HO-1 is a stress-inducible gene highly responsive not only to ROS but also to a large number of other internal and external factors causing cellular stress.15 It is therefore conceivable that adenoviral transduction causes cell stress which results in an increase in HO-1 mRNA. However, the change in HO-1 mRNA expression is significantly higher in Nrf2-transduced cells in comparison to respective LacZ-controls. Although we did not see a significant increase of NQO1 mRNA at 48 hours, the NQO1 mRNA as well as the other target genes were induced at 24 hours after transduction (supplemental Figure I). Also NQO1 protein was dose-dependently increased in HASMCs (Figure 1C). In Nrf2-transduced RaASMCs, the increase in the HO-1 expression with respective MOIs is less than in HASMCs because of their lower transduction efficiency. However, the HO-1 mRNA expression was significantly increased in comparison to LacZ control with MOI 500 (Figure 1D). AdNrf2 also increased the HO-1 activity is RaASMCs. With MOI 500, the increase in the activity was 2.2±0.7 fold in comparison to LacZ control (P=0.037).

Nrf2 Overexpression Inhibits HASMC and RaASMC Proliferation

Adenoviral overexpression of HO-1 has been shown to inhibit SMC growth in vitro.16 Inasmuch as Nrf2 gene transfer induced HO-1 expression in both HASMCs and RaASMCs, we hypothesized that Nrf2 overexpression would impact on SMC proliferation. In RaASMCs, AdNrf2 transduction significantly inhibited proliferation assessed by [3H]-thymidine incorporation (Figure 2A). To assess the role of HO-1 in the growth inhibitory effect of Nrf2 gene transfer, we used tin protoporphyrin IX to inhibit HO activity. 5 μmol/L tin protoporphyrin IX inhibited the activity by 64.9±18.7% (P=0.01) and 10 μmol/L by 51.2±14.3% (P=0.027). The inhibition of cell growth in RaASMCs was significantly reversed by the inhibitor (Figure 2B), showing that the growth inhibition was at least partially mediated by HO-1. Also in HASMCs, Nrf2 transduction inhibited proliferation. Thymidine incorporation was decreased by 15.3±2.6% (P=0.012) with MOI 100 and by 18.4±3.1% (P=0.027) with MOI 250 in Nrf2 transduced cells in comparison to LacZ controls.

AdNrf2 Increases the Expression of NQO1 and Decreases Oxidative Stress in Balloon Injured Rabbit Arteries In Vivo

After having established the ability of Nrf2 gene transfer to induce antioxidant enzymes in vitro, we next wanted to examine the impact of Nrf2 gene transfer on oxidative stress in vivo in the rabbit aortic balloon denudation model. To this end, the previously established rabbit model of balloon injury and intraarterial gene transfer13 was used. We used very clean clinical grade adenoviral vectors devoid of any contaminants. Furthermore, the viral dose had been optimized in our previous studies to avoid potential proinflammatory effects.5,13 AdNrf2 gene transfer resulted in transgene expression, which was detectable by RTPCR at 7 days and 14 days but no longer detectable at 28 days after transduction (Figure 3A). To assess the ability of Nrf2 gene transfer to induce antioxidant enzymes and to assess oxidative stress in the vessel wall, the expression of NQO1 and the amount of oxidatively modified LDL which readily accumulates in the injured rabbit vessels17 was determined by immunostaining. The number of NQO1 positive cells present in the intima was significantly higher in Nrf2 transduced vessels compared with LacZ controls 7 days and 14 days after gene transfer (Figure 3B and 3C). The NQO1 positive cells are localized near to the lumen of the aorta, and they colocalize with VSMCs identified as Nrf2 transduced cells at 28 days as the lesions matured and acquired a more organized appearance. NQO1 was constitutively expressed in medial SMCs (not shown), supporting the notion that NQO1 is constitutively expressed in quiescent, but not in phenotypically altered, proliferating SMCs.

To evaluate the extent of oxidative stress by determining LDL oxidation, antibodies against oxidatively-modified apoB (Ox4e6) or HNE-modified LDL (HNE7) were used. Nrf2 gene transfer significantly reduced the accumulation of oxidized LDL in the injured vessel wall assessed by both antibodies at 14 days after transduction (Figure 4A through 4D). The Ox4e6 positivity colocalized with macrophage-rich areas of the lesions, whereas HNE7 staining was more diffusely spread in the neointima (Figure 4B and 4D).

AdNrf2 Reduces Inflammation in Balloon Injured Rabbit Arteries but Has No Impact on Reendothelialization

Next we examined the effect of AdNrf2 gene transfer on injury-induced inflammation by immunostainings against a
macrophage-specific marker RAM-11 and a proinflammatory chemokine MCP-1. The number of macrophages normalized to the area was significantly smaller at 14 days in AdNrf2 transduced vessels in comparison to AdLacZ controls (Figure 5A and 5B). In addition, the staining for MCP-1, which was diffusely spread within neointima, was significantly reduced at 14 days in Nrf2 transduced aortas (Figure 5C and 5D). Nrf2 had no impact on reendothelialization assessed by the percentage CD31-positive endothelium of luminal circumference (43±7 versus 37±6 14 days and 59±19 versus 53±9 28 days after transduction in LacZ versus Nrf2-transduced vessels).

Nrf2 Gene Transfer Inhibits Neointimal SMC Proliferation With Concomitant Decrease in Apoptosis and No Net Effect on I/M Ratio

Nrf2 gene transfer inhibited VSMC proliferation in cultured human and rabbit VSMCs. To assess whether AdNrf2 is also able to inhibit cell proliferation in vivo, the injured aortas were immunostained against BrdU-labeled cells. The number of BrdU-positive cells in the intimal layer was significantly (P<0.05) reduced at 7 days and 14 days after transduction (Figure 6A and 6B). However, the number of apoptotic cells in the neointima was decreased concomitantly, reaching statistical significance at 14 days after transduction (Figure 6C and 6D). When assessing the intima area (not shown) or the I/M ratio, there was no change in Nrf2-transduced versus controls in any of the time points studied (Figure 6E and 6F). There were also no significant changes in other morphological parameters, including the luminal circumference and area, the medial area, and the circumference of internal or external elastic lamina (data not shown).

Discussion

Nrf2 has recently been shown to have antiinflammatory effects in the vasculature. In cultured endothelial cells, Nrf2 is activated by shear stress, a potent antiinflammatory force.18 Overexpression of Nrf2 downregulates the tumor necrosis factor (TNF)-α–induced expression of vascular cell adhesion molecule (VCAM)-1 at the transcriptional level.18,19 In addi-

Figure 3. The expression of human Nrf2 as determined by RT-PCR in injured rabbit aorta after gene transfer and the expression of the target gene NQO1 in the aortic wall A. Representative data showing the expression of human Nrf2 7 and 14 days after gene transfer. The size of the product is 802 bp. Lane 1: Molecular weight marker, lane 2: negative control, lane 3: 7 days after AdLacZ transfer, lane 4: 7 days after AdNrf2 transfer, lane 5: 14 days AdLacZ transfer, lane 6: 14 days after AdNrf2 transfer, lane 7: 28 days after AdLacZ transfer, lane 8: 28 days after AdNrf2 transfer, lane 9: Control without reverse transcriptase, lane 10: Positive control (human Nrf2 cDNA), lane 11: Molecular weight marker. B and C. The percentage of intimal cells expressing NQO1 and representative images from sections immunostained against NQO1. Original magnification 200×. Bars=100 μm. The data in bar graphs is depicted as mean percentage of NQO1 positive intimal cells±SEM. *P<0.05 and **P<0.01 compared with LacZ control.

Figure 4. Nrf2 gene transfer reduces immunoreactivity against oxidized LDL in the aortic wall. A and B. Staining with the antibody against oxidized apoB100 (mAb Ox4e6) and representative images from Ox4e6-immunostained sections at 14 days after gene transfer. C and D. Staining with the antibody recognizing hydroxynonenal-modified LDL (HNE7) and representative images at 14 days after gene transfer. The data in panels A and C are depicted mean percentage of the stained area±SEM; *P<0.05 compared with LacZ control.
tion, adenoviral Nrf2 overexpression protects from oxidant injury and inhibits monocyte adhesion in endothelial cells in vitro. Nrf2 mediates adaptive augmentation of antioxidant defenses of vascular cells on exposure to lipid oxidation products such as oxidized LDL, a lipid-derived aldehyde 4-hydroxynonenal, or cyclopentenone prostaglandins and isoprostanes. The impact of Nrf2 on vascular inflammatory processes in vivo has not been studied to date, but in the acute pleural inflammatory model, Nrf2 gene ablation has been shown to exacerbate inflammation. In addition, in the cutaneous wound repair model, which shares many similarities with the healing process after balloon injury, the expression of several important factors involved in wound healing was significantly reduced in early wounds of the Nrf2 knockout animals, and the late phase of repair was characterized by prolonged inflammation. Thus Nrf2-dependent antioxidant defenses appear to play a significant role in the inflammation in vascular as well as in other tissues. To our knowledge, our study is the first to show antioxidant and antiinflammatory effects of Nrf2 gene transfer in vivo in the vessel wall.

Figure 5. Nrf2 gene transfer reduces inflammation in Nrf2 transduced arteries. A, Inflammation in balloon-injured aorta after LacZ or Nrf2 gene transfer is assessed by counting the number of RAM-11-positive macrophages/mm² in the intima (mean ± SEM). *P < 0.05. B, Representative images from RAM-11 immunostained sections at 14 days after gene transfer. C, The expression of MCP-1 in injured aorta after LacZ or Nrf2 gene transfer assessed by percentage of positively stained area of the intima (mean ± SEM). *P < 0.05. D, Representative images from MCP-1 immunostained sections at 14 days after gene transfer. Original magnification 200×. Bars = 100 μm.

Figure 6. Nrf2 gene transfer reduces cell proliferation and apoptosis in injured aortas but has no effect on the intima/media (I/M) ratio. A, The percentage of proliferating cells in the intima after gene transfer with AdLacZ or AdNrf2. B, Representative images of sections immunostained for BrdU-labeled cells at 14 days after transduction. C, The percentage of apoptotic cells within the intima assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-staining after gene transfer of LacZ or Nrf2. D, representative images of TUNEL-stained sections at 14 days after gene transfer with AdLacZ or AdNrf2. Arrows indicate TUNEL-positive nuclei. E, I/M ratio of AdNrf2 or AdLacZ transduced vessels after gene transfer. Arrowheads indicate the internal elastic lamina. The data in panels A, C, and E are depicted as mean ± SEM; *P < 0.05 and **P < 0.01. Original magnification 200×. Bars = 100 μm.
In the present study, Nr2 gene transfer decreased oxidative stress assessed by the accumulation of oxLDL in the arteries of cholesterol-fed animals. This may be explained by the fact that Nr2 target genes having direct antioxidant functions limit the oxidation of LDL. Also the increased GSH synthetic capacity via induction of the rate-limiting enzyme GCL may impact on LDL oxidation, as GSH inhibits cell-mediated oxidation of LDL. In addition, Nr2 target genes may also include genes that are involved in hydrolysis of oxLDL. For example, in the gene expression analysis of Nr2-overexpressing human endothelial cells (Jyrkkänen et al, unpublished, 2005), aldose reductase and fatty aldehyde dehydrogenase, which catalyze decomposition of lipid-derived aldehydes and oxidized phospholipids, are induced by Nr2. Also several glutathione S-transferases which are involved in detoxification of short-chain aldehydes derived from lipid peroxidation are Nr2 target genes, and combined increase in their activity and GSH concentration thereby potentially limits oxLDL accumulation.

In this study, Nr2 inhibited VSMC proliferation through a mechanism partially dependent on HO activity. To our knowledge, this is the first study to show antiproliferative effects of Nr2. Previous studies have shown that HO-1 inhibits VSMC proliferation through multiple mechanisms. The end products of HO catalyzed reaction, carbon monoxide (CO), bilirubin, and biliverdin inhibit VSMC growth. Whether the growth inhibition of HO-1 or its catalytic products occurs through induction of apoptosis, inhibition of cell cycle progression, or both is unclear, but there is strong evidence that both CO and bilirubin inhibit VSMC proliferation by arresting the cell cycle progression at the G1 phase. At this juncture, it is of interest to note that the antiproliferative effects of proculol and rapamycin are at least partially mediated by HO-1. Besides HO-1, there are also other potential mechanisms by which Nr2 overexpression exerts its effects on VSMC growth. As exogenous ROS as well as ROS derived from the activation of NAD(P)H oxidases by various growth factors and cytokines contribute to the VSMC growth, it is conceivable that the augmentation of other antioxidant defenses such as the enhancement of the GSH synthetic capacity contribute to the growth inhibitory effects.

Despite the fact that Nr2 gene transfer was able to inhibit SMC proliferation in vitro and reduce the number of proliferating cells determined by BrdU staining in vivo, it had no impact on I/M ratio. This may be explained by the fact that also the number of apoptotic cells in neointima decreased in Nr2-transduced vessels. Although the role of apoptosis in neointimal growth is still somewhat controversial, it is generally accepted that the early wave of apoptosis of medial VSMCs occurring within hours after injury provokes a greater wound healing response thus exacerbating neointima lesion formation. However, the second wave of apoptosis confined to neointimal VSMCs and taking place days to weeks after injury may limit lesion growth and is thus considered beneficial. This notion is supported by studies in which gene transfer of proapoptotic proteins such as p53 or Fas ligand inhibit restenosis. It is therefore possible that Nr2, which has been shown to protect against apoptosis in vascular cells, may despite its antiinflammatory and antiproliferative effects have no impact on restenosis via inhibiting apoptosis of the intimal VSMCs needed for the appropriate remodeling of the injured vessel.

There is experimental evidence in the literature indicating that oxLDL could contribute to the pathophysiology of restenosis by, eg, promoting VSMC proliferation and migration via activation of the PDGF pathway. Inflammatory cells contribute to the stenotic process not only by producing ROS and oxidizing LDL, but also by releasing cytokines and other inflammatory factors promoting VSMC recruitment and proliferation. However, it should be noted that despite the similarities, atherosclerosis and restenosis are fundamentally different processes, oxLDL and macrophages having a key role in the former but not the latter. One of the earliest hallmarks of atherosclerosis is the accumulation of macrophage foam cells in the artery wall, whereas restenosis is primarily driven by VSMCs. We propose that the effect of Nr2 on VSMC apoptosis overrides the possible beneficial effects the reduction of inflammation and oxLDL accumulation could have on neointima formation. Furthermore, in our study, Nr2 transduction did not improve endothelial cell recovery. This may contribute to the lack of an effect on I/M ratio, as endothelium-derived NO is a critical factor suppressing the VSMC growth. The catheter-mediated gene transfer method used in this study does not effectively target endothelial cells in balloon-injured vessels, which is a limitation as Nr2 overexpression could potentially enhance reendothelialization of injured arteries. It is also noteworthy that the limited gene transfer efficiency of intraarterial gene transfer favors the therapeutic use of secreted proteins and other genes that transduce their effects outside the cell, such as nitric oxide synthase or HO-1. Although many target genes induced by Nr2 such as HO-1 have that potential, the transduction efficiency may be inadequate in the in vivo setting for therapeutic effects.

In summary, our results show that adenoviral gene delivery of Nr2, a transcription factor responsible for concerted induction of antioxidant and cytoprotective genes on oxidative or electrophilic stress, is able to induce antioxidant genes and inhibit proliferation in VSMCs in vitro. In addition, this study is the first to show in vivo that Nr2 gene transfer reduces inflammation and oxidative stress in the vessel wall. These results demonstrate the applicability of transcription factor gene therapy in vascular pathologies, in which oxidative stress plays an important role.

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Disclosures
None.
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13. Levonen et al Nrf2 Gene Transfer Reduces Oxidative Stress 747


24. Levonen et al Nrf2 Gene Transfer Reduces Oxidative Stress 747


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Supplemental Materials and Methods

Cloning and Production of Adenoviruses

Plasmid pAdCMV was constructed by cloning the fragment containing the CMV promoter, multiple cloning site and bovine growth hormone polyA tail (nucleotides 208-1439) from the pcDNA3 vector (Invitrogen) to the BglII restriction site of the pAdBglIII vector. The pAd-Nrf2 was cloned by excising and ligating the XhoI/XbaI fragment containing the human Nrf2 cDNA from pCI-Nrf2 into pAdCMV. This construct was used to generate the recombinant adenovirus using standard techniques. The LacZ-expressing adenovirus was used as a control.

Cell Culture and Viral Transduction

Human aortic smooth muscle cells (HASMCs) (Cascade Biologics) were maintained in 231 medium supplemented with Smooth Muscle Growth Supplement (both from Cascade Biologics). Rabbit aortic smooth muscle cells (RaASMCs) were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

HASMCs and RaASMCs were seeded onto 35-mm dishes at the density of 100000 cells/dish and allowed to adhere for 24 h. Cells were transduced in serum-free conditions with AdNrf2 or AdLacZ with different multiplicities of infection (MOI). After an hour, normal cell culture supplements were added and cell culture continued for additional 16 h. Cells were washed with Phosphate Buffered Saline (PBS, Gibco BRL) and fresh cell culture medium was added. Cells were harvested after 48 h for real time-PCR and Western Blot analyses. Each experiment was performed in triplicate wells and repeated 2-3 times.
RNA Isolation and Quantitative Real-time PCR

HASMCs or RaASMCs transduced with AdNrf2 or AdLacZ were collected and RNA extracted with Trizol-reagent (Invitrogen). 2 µg of total RNA was used for the cDNA synthesis using random hexamer primers (Promega) and M-MuLV reverse transcriptase (New England Biolabs). The relative expression levels of mRNA encoding Nrf2, HO-1, GCLC, GCLM and NQO1 in HASMCs and HO-1 in RaASMCs were measured according to the manufacturer’s protocol by quantitative RT-PCR (ABI PRISM 7700 Sequence detector, Applied Biosystems) using specific Assays-on-Demand target mixes for human Nrf2 (Assay ID: Hs00232352_m1), human HO-1 (Hs00157965_m1), human GCLM (Hs00157694_m1), human GCLC (Hs00155249_m1) and human NQO1 (Hs00168547_m1) or Assays-by-Design (Applied Biosystems) target mix for rabbit HO-1. The forward and reverse primers designed for rabbit HO-1 were 5'-CCTCCCTGTACCACATCTACGT-3' and 5'-AGCTCCTCCGGGAAGTAGAG-3', respectively. The expression levels were normalized to ribosomal RNA and presented as fold change in the expression vs. non-transduced control.

Western Blotting

Cells were lysed and 10 µg of total protein was used for standard SDS-PAGE electrophoresis. The proteins were transferred to nitrocellulose membranes which were incubated with the following primary antibodies: rabbit polyclonal anti-Nrf2 (Santa Cruz), rabbit polyclonal anti-HO-1 (Stressgen), anti-GCLM and -GCLC (gifts from Dr. Terrance Kavanagh, University of Washington, Seattle, WA), mouse monoclonal anti-NQO1 (Clones A180 and B771, gifts from Dr. David Ross, University of Colorado, Denver, CO) and rabbit polyclonal anti β-actin (Cell Signaling Technology). Blots were visualized using HRP-conjugated secondary antibodies and Supersignal™ chemiluminescence substrate (Pierce, Rockford, IL). Densitometric analysis of western blots was
performed with NIH ImageJ software. The intensities of Nrf2, HO-1, GCLM, GCLC and NQO1 bands were measured from 3 blots and results were normalized to β-actin expression.

**Cell Proliferation Assay**

HASMCs and RaASMCs were seeded onto 12-well plates at 30000 or 10000 cells/well, respectively. Sixteen hours after transduction the growth medium was changed to basal medium supplemented with 0.5 % FBS for 24 h (HASMCs) or 48 h (RaASMCs), after which normal cell culture supplements were added 24 h prior to harvesting. Newly synthesized DNA was labeled with [3H]-thymidine (1 µCi/ml) (Amersham Biosciences, Buckinghamshire, UK) for 4 h, cells were washed with PBS and lysed with 0.2 N NaOH. The activity of [3H]-thymidine was measured and normalized to total protein. For the blocking studies, 5 or 10 µM of HO inhibitor Sn(IV)Protoporphyrin IX dichloride (Frontier Scientific) was added 24 h prior harvesting. Each experiment was performed in triplicate and repeated three times.

**HO-1 Activity Assay**

The activity of HO-1 was measured by quantifying the release of bilirubin into the culture media as described previously. RaASMCs were transduced with AdLacZ or AdNrf2 with MOI 500. 24 hours after transduction, 0-10 µM Sn(IV)Protoporphyrin IX dichloride (Frontier Scientific) was added to the media. 48 h after transduction the culture media was collected and 0.5 ml of the medium was combined with 250 mg of barium chloride (Sigma) and 0.75 ml of benzene (Merck). The benzene layer was separated from aqueous layer by centrifugation at 13000 g for 30 min. The bilirubin was determined spectrophotometrically as a difference in absorbance between 450 nm and 600 nm using a molar extinction coefficient of 27.3 mM⁻¹ cm⁻¹.
Animal Experiments

35 male New Zealand White rabbits were fed a 0.25 % cholesterol diet for 2 weeks before the
denudation of the descending aorta as in\textsuperscript{8}, using 3.0F arterial embolectomy catheter (Sorin
Biomedical). Three days later, the gene transfer of AdNrf2 or AdLacZ was performed as described
previously\textsuperscript{8}, using a viral dose of 1.5 x 10\textsuperscript{10} pfu. Intravascular procedures were performed during
ketamine/medetomidine anesthesia. Animals received preoperatively glycopyrrone and kefuroxim
and postoperatively carprofen. Animals were sacrificed 7, 14 or 28 days after gene transfer. The
number of animals in each group was as follows: animals sacrificed 7 d after gene transfer, 6
animals in both groups; animals sacrificed 14 d after gene transfer, 7 animals in both groups;
animals sacrificed 28 d after gene transfer, 4 animals in AdlacZ and 5 animals in AdNrf2 group.
Tissue samples were collected from the transduced part of the aorta. All animal experiments were
approved by the Experimental Animal Committee of the University of Kuopio.

Histological Analysis

Three hours before sacrifice, the animals were injected I.V. with 50 mg of bromodeoxyuridine
(BrdU) dissolved in 40 % ethanol. After sacrifice, the transduced part of aorta was removed, flushed
gently with saline and divided in 4 equal parts for histological paraffin sections, cryosections and
snap-frozen samples for RNA extraction and gene expression analysis as described previously.\textsuperscript{8}
Paraffin sections were stained with hematoxylin-eosin for intima/media (I/M) analysis.
Immunohistochemical stainings were performed for the detection of NQO1 (Clones A180 and
B771), oxidized lipids (mAb 4e6\textsuperscript{8} and HNE\textsuperscript{7}), macrophages (RAM-11; DAKO), endothelium
(CD31; DAKO), β-galactosidase (anti β-gal; Promega) and BrdU-positive cells (Bu20a; DAKO)
from paraffin sections, and MCP-1 (AF-279-NA; R&D Systems) from cryosections. An avidin-
biotin-horseradish peroxidase system (Vector Laboratories) and DAB Plus-kit (Zymed
Laboratories) were used for signal detection. Control immunostainings were conducted without the
primary antibodies. Detection of apoptotic cells was performed with ApopTag-kit, which detects the DNA cleavage in apoptotic cells using the TUNEL assay, according to manufacturer's instructions (Intergen Company). Morphometry and analysis of I/M ratio was done using Olympus AX-70 microscope (Olympus Optical) and AnalySIS software (Soft Imaging Systems).

RNA Isolation and RT-PCR for Detection of Nrf2

Total RNA extracted from the aorta was used for the cDNA synthesis as in. The cDNA was amplified using DyNAzyme™ EXT polymerase (Finnzymes, Espoo, Finland) and primers specific for human Nrf2 sequence as follows: forward: 5'-CAGGCTCAGTCACCTGAAACTTCT-3' and reverse: 5'-TCTCTGGTGTGTTCTCACATTGGG-3'. Hot start at +94 °C was followed by initial denaturation of 3 minutes in 80 °C. The reaction was subjected to 30 cycles of 30 s denaturation in 94 °C, 40 s annealing in 57 °C and 1 min extension in 72 °C. Extension in final cycle was 5 min.

Statistical Analysis

GraphPad Prism 4 (GraphPad Software Inc.) software was used for statistical analysis. Student's t-test was used to evaluate statistical significances and a value of p < 0.05 was considered statistically significant. Numerical values for each measurement are shown as mean ± SEM.
Supplemental References


**Supplemental Figure Legends**

**Supplemental Figure I.** The mRNA expression of heme oxygenase-1 (HO-1), glutamate-cysteine ligase modifier subunit (GCLM), glutamate-cysteine ligase catalytic subunit (GCLC), and NAD(P)H:quinone oxidoreductase-1 (NQO1) from AdNrf2 or AdLacZ transduced human aortic smooth muscle cells (HASMC) 24 h after transduction. The data is derived from two experiments done in triplicate wells and presented as mean ± SEM. *, p < 0.05 in comparison to non-transduced control and #, p < 0.05 as compared to respective MOI of AdLacZ.

**Supplemental Figure II.** Densitometric analysis of the protein expression of Nrf2 (A), HO-1 (B), GCLM (C), GCLC (D) and NQO1 (E) from AdNrf2 or AdLacZ transduced HASMC, presented as relative expression normalized to β-actin. Densitometric data is derived from two experiments performed in triplicate and presented as mean ± SEM. *, p < 0.05 in comparison to non-transduced control and #, p < 0.05 as compared to respective MOI of AdLacZ.
Supplemental Figure II

A. Relative Nrf2 expression

B. Relative HO-1 expression

C. Relative GCLM expression

D. Relative GCLC expression

E. Relative NQO1 expression