KLF2 primes the antioxidant transcription factor Nrf2 for activation in endothelial cells

Reinier A. Boon,1* Joost O. Fledderus,1* Oscar L. Volger,1,3 Hanna Hurttila,2 Seppo Ylä-Herttuala,2 Hans Pannekoek,1 Anna-Liisa Levonen2 and Anton J.G. Horrevoets3

From the 1Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands, the 2Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute, University of Kuopio, Kuopio, Finland, and the 3Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands

*These authors contributed equally to this work.

Chapter 5
Abstract

Atheroprotective blood flow induces expression of anti-inflammatory Krüppel-like factor 2 (KLF2) and activates antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in vascular endothelium. Previously, we obtained KLF2-induced gene expression profiles in ECs, containing several Nrf2 target genes. Our aim was to investigate the role of KLF2 in shear stress-mediated activation of Nrf2 in human umbilical vein endothelial cells (HUVECs).

Expression of Nrf2 and its targets NAD(P)H dehydrogenase quinone 1 (NQO1) and heme oxygenase (HO-1) was elevated by shear and KLF2. KLF2 knockdown showed that shear-induced expression of NQO1 but not Nrf2 was dependent on KLF2. KLF2 overexpression in absence of flow resulted in more efficient activation of Nrf2 by tert-butyl hydroquinone (tBHQ) through enhanced nuclear localization, and promoted expression of a large panel of Nrf2-dependent genes resulting in superior protection against oxidative stress. Comparison of shear-, KLF2- and Nrf2-induced transcriptomes showed that the majority of shear-modulated gene sets is influenced by KLF2 and/or Nrf2.

We report that KLF2 substantially enhances antioxidant activity of Nrf2 by increasing its nuclear localization and activation. The synergistic activity of these two transcription factors forms a major contribution to the shear stress-elicited transcriptome in endothelial cells.
Chapter 5

Introduction

The arterial endothelium is profoundly affected by fluid shear stress and the resulting effect on the signalling and transcriptional networks of endothelial cells (ECs) are thought to lie at the basis of the focal development of atherosclerotic plaques. The largest part of the endothelium is exposed to a laminar blood flow and high shear stress levels, which, when present for prolonged periods of time, have been shown to activate protective mechanisms in ECs that preclude atherosclerotic plaque formation. Oscillatory or even absence of shear stress in local areas of disturbed blood flow enables detrimental atherogenic effects such as inflammation and oxidative stress to take full effect. An important protective mechanism is mediated by the shear-responsive transcription factor KLF2. In recent years KLF2 is emerging as a master regulator of endothelial quiescence, anti-inflammatory and anti-thrombotic properties and vascular tone by activating atheroprotective and inhibiting atherogenic transcription.

We have previously studied genome-wide transcriptional effects of prolonged shear stress and inflammatory stimuli, where we divided co-ordinately regulated genes into three groups and found several conserved transcription factor binding elements commonly present in the promoters of the genes in each group. In that study, we could show that shear stress and KLF2 inhibit nuclear activity of activating transcription factor 2 (ATF2), thereby suppressing pro-inflammatory gene induction by tumor necrosis factor alpha (TNFα). The second gene cluster, which was inhibited by shear stress, showed over-representation of SMAD binding elements that are occupied by SMAD3/4, which together with AP-1 drives TGF-β-induced transcription. KLF2 induces the inhibitory SMAD7 and reduces nuclear levels of the AP-1 member c-Jun, thereby fully suppressing TGF-β driven gene expression. A third cluster showed enrichment for the antioxidant response element (ARE), suggesting a role for nuclear factor erythroid 2-like 2 (Nrf2) that binds the ARE.

Shear stress activates a protective ARE-dependent transcriptional program through coordinate induction of detoxifying phase II enzymes and antioxidant enzymes that are crucial in the cellular defence against electrophilic compounds and reactive oxygen species (ROS). The molecular sensor for electrophiles and ROS in the cell is thought to be Keap1 (Kelch-like ECH-associated protein 1), which acts as an inhibitor by binding Nrf2 and the actin cytoskeleton, thereby keeping it sequestered in the cytoplasm and promoting its degradation by the proteasome. Modification by electrophiles of cysteine residues in Keap1 disrupts its ability to target Nrf2 to the proteasome, thereby allowing nuclear accumulation of Nrf2 and ARE-dependent gene expression. Several groups have now reported that atheroprotective levels of shear stress induces activation and nuclear translocation of Nrf2, as well as Nrf2-dependent cytoprotective gene expression in endothelial cells in vitro and that this effect can be blocked by reducing oxidative stress levels with ROS scavengers. Furthermore, activation of the Nrf2/ARE pathway inhibited TNFα-induced inflammation in endothelial cells and Nrf2 gene transfer has anti-inflammatory and antioxidant effects in the vessel.
wall in vivo. Other in vivo studies established an important link between antioxidant gene expression patterns and arterial regions susceptible to formation of atherosclerotic plaques and Dai and co-workers demonstrated increased nuclear Nrf2 protein levels in endothelial cells in atheroprotected regions of the mouse aorta.

Interestingly, our studies of human umbilical vein endothelial cells (HUVECs) overexpressing KLF2 showed that KLF2 upregulates several phase II detoxifying enzymes and antioxidant genes, including NQO1, HO-1, glutamate-cysteine ligase modifier subunit (GCLM) and catalase (CAT), which are all known target genes of Nrf2. We decided to study the role of KLF2 in the shear stress-induced activation of Nrf2 and utilized novel strategies for identifying Nrf2 target genes and compared this with shear- and KLF2-controlled expression profiles. We conclude that, although KLF2 and Nrf2 play individual roles in the shear-induced transcriptome, there is a much stronger detoxifying and antioxidant response in the presence of KLF2 as a result of priming of Nrf2 activation through enhanced nuclear localization.
Methods

Reagents
Tert-butyl hydroquinone (tBHQ) and tert-butyl hydroperoxide (tbH2O2) were purchased from Sigma-Aldrich (St Louis, MO), dissolved in the vehicle dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS), respectively.

Cell culture and shear stress
HUVECs were isolated and cultured in Medium-199 (M199, Invitrogen, Carlsbad, CA), supplemented with 20% (vol/vol) fetal bovine serum (FBS), 50 μg/mL heparin (Sigma-Aldrich), 6-25 μg/mL endothelial cell growth supplement (ECGS; Sigma-Aldrich), and 100 U/mL penicillin/streptomycin (Invitrogen). Long term shear stress exposure (6 days) was performed as previously described. After a 24-hour period to allow attachment of the endothelial cells to the fibronectin coated capillaries, flow was gradually increased to a pulsatile shear stress of 19 ± 12 dynes/cm², which was maintained over the next 6 days with intermediary medium changes. For static controls, HUVECs from the same isolate were seeded in fibronectin-coated cell culture flasks and grown to confluency. Total RNA was extracted using Trizol reagent (Invitrogen).

siRNA mediated knockdown and lentiviral overexpression
Stable overexpression of KLF2 and Nrf2 in HUVECs for at least 5 days was achieved by transducing HUVECs with lentiviral vectors expressing KLF2 or Nrf2 cDNA4. For KLF2, stable knockdown was achieved using a lentivirally delivered specific short hairpin RNA sequence directed against KLF2. Lentiviral experiments included control cells that were transduced with an empty vector or a non-silencing shRNA control vector. Nrf2 knockdown was done with Nrf2 siRNA (SC-37030; Santa Cruz Biotechnology, Santa Cruz, CA) that was transfected into HUVECs using Oligofectamine reagent (Invitrogen) as described before.

Immunofluorescence
Fluorescent imaging of Nrf2 protein in mock- and KLF2 transduced HUVECs was performed as described.6 The Nrf2 antibody (SC-722) was purchased from Santa Cruz Biotechnology.

Viability assay and ROS measurement
Freshly isolated HUVECs were transfected with non-specific or Nrf2 siRNA and cultured for 24 hours, or transfected with mock or KLF2 lentivirus and cultured for 4 days and then pretreated for 24 hours with 20 μM tBHQ or vehicle (DMSO). Following pretreatment, cells were stimulated for 30 minutes with 200 μM tbH2O2, washed once with Hank’s balanced salt solution (HBSS), incubated in fresh culture medium and allowed to recover for 6 hours (viability assay) or 3 hours (photographs and ROS measurement). To determine cellular viability, cells were washed with HBSS after the recovery period and subsequently incubated in normal culture medium with the addition of 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). After 2 hours, medium was discarded and the formazan crystals that formed were dissolved in isopropanol, and optical density was measured in triplicate at 590 nm with reference at 650 nm. For the ROS measurement, cells were washed once with HBSS and incubated with 10 μM 5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate (Invitrogen) in HBSS for 30 minutes at 37°C. Hereafter, the dye solution was removed and cells were incubated in fresh medium for 30 minutes at
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37°C. Finally, the cells were washed once more with HBSS and fluorescence was measured (λexcitation = 485 nm and λemission = 530 nm) on a Varioskan Flash spectral scanning multimode reader (Thermo Electron Corporation, Breda, The Netherlands).

Real-time reverse transcriptase polymerase chain reaction
Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed as previously described.3 In brief, cDNA from 0.5-1 μg of total RNA was synthesized with Superscript II reverse transcriptase (Invitrogen) according to manufacturers protocol and diluted 10x for gene-specific analysis with real-time PCR. All PCR reactions were performed in a 15 μl reaction on an iCycler thermal cycler system (Biorad Laboratories, Veenendaal, Netherlands). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). After correction for the house-keeping control large ribosomal phosphoprotein P0, the mRNA levels were expressed as ratios compared to unstimulated or mock transduced control cultures.

Microarray experiments and analyses
Probe synthesis, hybridization, feature extraction and normalization by linear correction methods were all performed as described.3 Normalized data were stored in our Rosetta Resolver database (Rosetta Biosoftware, Seattle, WA). Gene set enrichment analysis (GSEA) was done with GSEA version 2.0 using 10log ratios exported from Rosetta. Analyses were performed with 10 permutations with a gene set permutation type. Output lists were ranked based on the normalized enrichment score. The ranking, nominal p-value and FDR q-value for individual gene sets was used to compare results between experimental conditions. For transcription factor binding site analysis, the rVISTA and Genomatix MatInspector algorithms were fed with the gene symbols or Entrez GeneID of the top 50 genes from the ranked genelist for Nrf2 versus mock overexpression in HUVEC. In rVISTA, the Human March 2006 (hg18) assembly conserved in the alignment with the Mouse February 2006 (mm8) assembly was used to calculate over-represented transcription factor binding sites (TFBS) located in 500 basepair upstream regions of the 50 genes and using all upstream regions of human refseq genes as outgroup. The resulting list of enriched TFBS was queried to determine which of the 50 genes had a NFE2 binding site. In MatInspector, the top 50 genes were searched for common TFBS and positive hits in both DNA strands for the matrix names V$AP1R/V$NFE2.01 and V$AP1R/V$NFE2L2.01 were selected.

Statistical analysis
Experimental data are shown as mean of normalized ratios ± standard error of the mean (SEM) for the indicated number of experiments. Unpaired Student’s t-tests or –where indicated– one-way ANOVA with Bonferroni correction were used to calculate statistical significance. P-values of less than 0.05 were considered statistically significant.
Results

*KLF2 contributes to shear-mediated induction of NQO1 and HO-1*

We observed that prolonged exposure of HUVECs to shear stress potently induced mRNA expression of Nrf2. This increase was most likely followed by an increase in functional Nrf2 protein because NQO1 and HO-1, whose expression has been shown to depend on direct interaction of Nrf2 with the ARE in their promoter\(^{18,19}\) were concomitantly induced (Figure 1A). Based on this observation, a possible role of KLF2 in the shear-mediated induction of Nrf2 and its downstream genes was investigated. First, stable overexpression of KLF2 in static HUVECs for 7 days caused a significant 1.9-fold increase in Nrf2 expression and had effects of similar magnitude on NQO1 and HO-1 (Figure 1B). The effect on NQO1

![Figure 1](image)

*Figure 1. KLF2 and shear stress induce Nrf2 and its target genes NQO1 and HO-1.*

(A) Relative mRNA levels were measured in 3 different isolates of HUVECs exposed to shear stress or static conditions. (B) Expression of KLF2, Nrf2, NQO1 and HO-1 mRNA levels in HUVECs overexpressing KLF2 relative to mock. Expression of NQO1 (C) in mock and KLF2-transduced HUVECs treated for 48 hours with an siRNA directed against Nrf2 (grey bars) or a non-specific sequence (black bars) (*P<0.05). (D) Nrf2, NQO1, HO-1 and KLF2 expression levels were measured in static or sheared HUVECs during mock- or KLF2 knockdown. Statistical significance between the “shear mock” and “shear siKLF2” as measured by one-way ANOVA is indicated (***P<0.001; N.S. P>0.05).
Figure 2. KLF2 primes the activation of the Nrf2 pathway by inducing nuclear localization of Nrf2.
(A) Relative NQO1 mRNA levels in mock- and KLF2-transduced HUVECs treated for 24 hours with 20μM tBHQ (grey) or DMSO (black). (B) Cellular localization of Nrf2 protein (red) in HUVECs treated for 4 hours with 20μM tBHQ or vehicle. Nuclei were stained with Hoechst33342 (blue). The scale bars represents 10μm. (C) Nrf2-positive nuclei were scored in 4 separate photomicrographs. (D) Western blot showing Nrf2 protein levels in cytosolic and nuclear extracts from mock- and KLF2-transduced HUVECs treated for 4 hours with 20μM tBHQ or vehicle. (E) Nrf2 bands were quantified and normalized using α-Tubulin (cytosolic) and Lamin B (nuclear) signals. Normalized data for each of the four conditions are represented as nuclear to cytosolic Nrf2 ratios in arbitrary units (A.U.). *P<0.05; **P<0.01
was Nrf2-dependent as Nrf2 knockdown (Supplemental Figure I) abrogated KLF2-induced NQO1 expression (Figure 1C). Moreover, Nrf2 knockdown strongly decreased basal NQO1 expression, indicating that Nrf2 is constitutively active in HUVECs cultured under static conditions. Together, these results indicate that KLF2 causes a small, but significant induction of Nrf2 which contributes to expression of NQO1 and HO-1.

Next, we determined the contribution of KLF2 to shear-induced activation of Nrf2 by exposing HUVECs to prolonged shear stress during stable KLF2 knockdown. Lentivirally delivered KLF2 shRNA, inhibited KLF2 mRNA by 50-60% compared to an empty lentiviral vector (Figure 1D) or a non-silencing siRNA control vector (Supplemental Figure II). Although KLF2 knockdown under shear did not affect Nrf2 mRNA, it reduced expression levels of NQO1 (P<0.01) and showed a decreasing trend in HO-1 expression compared to a mock vector (Figure 1D). This finding suggests that KLF2 contributes to, but is not completely responsible for the expression of Nrf2 target genes by shear stress. An additional mechanism must be present by which shear stress fully activates NQO1 and HO-1 expression, possibly through post-translational activation of Nrf2 protein.

**KLF2 augments tBHQ-mediated Nrf2 activation**

Nrf2 can be activated by several chemical compounds, including sulforaphane (SFN) and tert-butyl hydroquinone (tBHQ), which have been used to study protection to oxidative stress and ARE-driven gene expression in many cell types. In HUVECs, NQO1 was induced by both tBHQ and SFN (Supplemental Figure IIIA). Although tBHQ resulted in a robust induction of NQO1, Nrf2 mRNA levels were unchanged, suggesting that tBHQ indeed promotes the activation of Nrf2 rather than increasing its expression (Supplemental Figure IIIB). The tBHQ-mediated induction of NQO1 was abrogated by Nrf2 siRNA, demonstrating that tBHQ induces NQO1 through activation of Nrf2 (Supplemental Figure IIIIC). Adding tBHQ under KLF2 overexpression resulted in an enhanced absolute expression level of NQO1 but did not affect the relative magnitude of induction (Figure 2A). To study how KLF2 induces Nrf2 activity, mock- and KLF2-transduced ECs were treated with tBHQ or vehicle for 4 hours. Immunofluorescence analysis of Nrf2 revealed that tBHQ induces nuclear accumulation of Nrf2 protein both in the presence and absence of KLF2. Levels of nuclear Nrf2 were higher in KLF2-transduced ECs, when compared to mock-transduced cells (Figure 2B-C). In a parallel experiment, nuclear and cytoplasmic Nrf2 levels were quantified by Western blotting analysis, confirming an increased nuclear localization of Nrf2 in KLF2-transduced cells (Figure 2D-E). The increased cytoplasmic stability of Nrf2 observed with tBHQ in mock cells and the concomitant increase in nuclear Nrf2 is in accordance with previously published reports. Remarkably, in KLF2 transduced endothelial cells where Nrf2 is already nuclear, tBHQ does not lead to increased cytoplasmic protein levels of Nrf2, but only to a relatively increased nuclear fraction. This observation demonstrates that stabilization and nuclear activation of Nrf2 by tBHQ seem to be two separate processes.
KLF2 primes Nrf2 for activation

To determine the functional effect of Nrf2 activation, we challenged HUVECs with an oxidative stress by stimulation with tert-butyl hydroperoxide (tBHQ). A 30-minute exposure to 200 μM of tBHQ caused cell death and disruption of the monolayer, which was completely rescued by pretreatment with tBHQ (Figure 3A, left panels). When Nrf2 was knocked down, tBHQ was no longer able to rescue the cells (Figure 3A, right panels). These results show that the protective effects of tBHQ are dependent on Nrf2. Next, we measured ROS levels and determined cell viability in mock- and KLF2-transduced HUVECs, pretreated with tBHQ or vehicle (DMSO) and subsequently stimulated with tBHQ (Figure 3B). In unstimulated conditions, KLF2 overexpression significantly increases cell viability. However, this does not confer increased protection against an oxidative insult, as exposure of KLF2-transduced cells to tBHQ exacerbates loss of cell viability compared to mock transduction. In support of this, ROS levels after tBHQ treatment are much higher in KLF2 expressing cells than in mock cells. Pretreatment with tBHQ increases cell viability and renders complete protection of both mock- and KLF2-transduced cells to tBHQ in terms of cell viability, accompanied by a reduction of ROS levels to below those measured in unstimulated mock cells. Figure 3C shows representative photomicrographs of the cells used in the experiments of Figure 3B.

The Nrf2-regulated transcriptome is primed by KLF2 and shear stress

To identify Nrf2 transcriptional targets in HUVECs, we lentivirally overexpressed Nrf2. Functional Nrf2 overexpression in 8 different isolates was first confirmed by RT-PCR for both Nrf2 and NQO1 (Supplemental Figure IV), followed by microarray expression profiling and Gene Set Enrichment Analysis (GSEA). Several previously reported Nrf2 targets as well as many novel candidate target genes are present in the top 50 of genes induced by Nrf2 compared to mock (Supplemental Table I). Moreover, promoter analysis revealed the presence of an ARE in the promoter of many of the top 50 and almost all of the top 10 genes. As expected, Nrf2 overexpression significantly upregulated the Nrf2-induced gene set that was derived from published records of Nrf2 downstream genes (Table 1).

The potentiating effect of KLF2 on the tBHQ-induced Nrf2-dependent gene expression was next studied at the genome-wide level. HUVECs overexpressing KLF2 and control cells were stimulated for 24 hours with 20μM tBHQ or vehicle (DMSO) and expression profiles were generated by microarray. Array data were analyzed using GSEA, specifically focussing on a literature-based Nrf2-induced gene set and 3 gene sets based on the presence of Nrf2 binding sequences in their promoter (Supplemental Table II). Stimulation with tBHQ clearly leads to a significant upregulation of the Nrf2-induced gene set under both control and KLF2 overexpression conditions (Table 1). GSEA revealed a major overlap for Nrf2 target genes that were upregulated by tBHQ as well as lentiviral Nrf2 overexpression (Supplemental Figure V). In addition, more than twice as many Nrf2-dependent genes were enriched by tBHQ under KLF2 than under mock conditions (Supplemental Table III) and tBHQ-induced
Figure 3. tBHQ protects against oxidative stress through Nrf2, especially in the presence of KLF2.

(A) HUVECs were transfected with either non-specific or Nrf2 siRNA, pretreated with vehicle (DMSO) or 20 μM tBHQ and subsequently exposed for 30 minutes to oxidative stress with 200 μM tbH₂O₂. (B) HUVECs were transduced with mock (white bars) or KLF2 (black bars) lentivirus for 4 days, pretreated with 20 μM tBHQ or vehicle (DMSO) and exposed to 200 μM tbH₂O₂ or PBS for 30 minutes. Statistical significance vs. the untreated mock control as measured by one-way ANOVA is indicated (**P<0.001). (C) Representative phase-contrast microscope photographs of the cells used in the experiment described in (B).
Figure 4. Nrf2-dependent genes upregulated by tBHQ under mock or KLF2 overexpression.

Treatment with tBHQ under KLF2 or mock conditions upregulated a specific Nrf2-induced gene set. Projected onto pathways for glutathione homeostasis (A), detoxification of H2O2 and iron homeostasis (B) and NADPH homeostasis (C) are core enriched genes in the Nrf2-induced gene set that was upregulated by tBHQ in control or KLF2 overexpressing endothelial cells. The color of the boxes indicates upregulation by tBHQ under mock only (yellow), KLF2 only (red) or both (orange) conditions.
activation of detoxifying and antioxidant pathways is greatly enhanced by KLF2 compared to mock (Figure 4). The Nrf2-induced gene set was also significantly upregulated by shear stress and KLF2 overexpression compared to controls (Table 1). In addition, the Nrf2 motif gene sets 1 and 2 (ARE sequence variants) received a much higher ranking and smaller nominal p-value with tBHQ treatment in KLF2 overexpressing cells (Table 1), indicating that the potentiating effects of KLF2 are general for genes with a Nrf2 binding site in their promoter and are not restricted to selected single genes. The third so-called Nrf2 motif gene set present in TRANSFAC, turned out to correspond to the binding motif for nuclear respiratory factor 2. This motif was not enriched underscoring the significance and specificity for Nrf2/ARE priming by KLF2 and shear stress (Table 1).

**Role of KLF2 and Nrf2 in the shear stress-modulated endothelial gene expression profile**

Next, we analysed our combined transcriptome database to dissect the roles of KLF2 and Nrf2 in shear stress-modulated gene expression, by comparing differential gene expression at both single gene and geneset level from the shear-, KLF2- and Nrf2-expression profiles. Overlap between expression profiles was limited when looking at single genes, selected for the individual conditions by rigid statistical cut-off in Rosetta Resolver (p<0.05), suggesting that the three conditions elicit mostly distinct expression profiles (Figure 5A). However, considerable overlap exists between gene sets upregulated by either shear, KLF2 and Nrf2 as studied by non-parametric GSEA. The number of gene sets upregulated by shear stress versus static was 582 out a total of 1765. About 76% of these 582 gene sets were also upregulated by overexpression of KLF2 and Nrf2 versus mock (Figure 5B). These observations imply that both transcription factors contribute to the majority of the functional transcriptional networks activated by shear stress, even though these transcription factors govern largely independent transcriptomes (Fig 5C).
Table 1. Upregulation of NRF2 gene sets by different conditions.

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<th>dbHQ vs VEHICLE (in KLF2 cells)</th>
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Table 1. Upregulation of NRF2 gene sets by different conditions.

For each gene set, its ranking based on the normalized enrichment score and the corresponding nominal p-value in the list of upregulated gene sets is indicated for 5 comparisons. NR indicates not ranked. Special nucleotide abbreviations: S=C/G, M=A/C, K=G/T, N=A/C/T/G.
Figure 5. Comparison of upregulated genes and gene sets between 3 transcriptomes.
Venn diagram representations of upregulated single genes with a Rosetta p<0.05 (A) or GSEA gene sets (B) in the comparisons shear stress versus static (SHEAR), KLF2 versus mock (KLF2) and Nrf2 versus mock (Nrf2). Overlapping gene sets are numbered A, B and C, which correspond to the schematic representation of the overall transcriptional effects (C).
Discussion

Shear stress has been established as a major factor controlling the transcriptome and thus phenotype of vascular endothelium by suppressing the pro-inflammatory and promoting the antioxidant response. We and others previously showed that KLF2, a transcription factor that is induced by shear stress, promotes a healthy endothelium by keeping it in a quiescent state and bestowing upon it anti-thrombotic, vasorelaxing and anti-inflammatory properties.\textsuperscript{3-5,23} KLF2 is thus an effector of shear-induced inhibition of the transcriptional activity of pro-inflammatory factors like ATF2, c-Jun and Smad3/4.\textsuperscript{3,6} In the present study we report that KLF2 is required for full shear stress-mediated Nrf2 activation and concomitant ARE-dependent target gene expression.

The moderate effects of KLF2 overexpression alone on Nrf2 transcription and protein activity could not fully explain the large increase of NQO1 and HO-1 caused by shear stress, even though KLF2 knockdown diminishes shear stress-mediated induction of NQO1 (Figure 1D). This indicates that even in the presence of KLF2, a specific Nrf2-activating stimulus is needed for full transcriptome effects, most probably represented by ROS which are known to be induced by shear stress.\textsuperscript{24} We investigated this in more detail by using tBHQ, a chemical known to activate Nrf2 and drive ARE-dependent gene expression. Our results show that tBHQ protects HUVECs from an oxidative insult in a Nrf2-dependent manner (Figure 3A). Treatment of KLF2-transduced HUVECs with tBHQ led to higher nuclear activity of Nrf2 compared to tBHQ-treated mock cells, resulting in superior NQO1 expression (Figure 2A). This result suggests that KLF2 primes the tBHQ-mediated activation of Nrf2. The notion that KLF2 helps to activate Nrf2 was further supported by analysis of microarray data from HUVECs treated with tBHQ under mock or KLF2 overexpression which revealed that under KLF2, tBHQ stimulation results in much higher ranking and smaller FDR q-value of Nrf2-dependent gene sets than under mock conditions (Table 1).

Functional relevance of these findings was demonstrated using ROS measurement and a viability assay. KLF2 overexpressing cells displayed an increased viability compared to mock control cells. Surprisingly, when challenged with tbH\textsubscript{2}O\textsubscript{2}, KLF2 overexpressing cells exhibited a dramatic increase in intracellular ROS levels and a concomitant decrease in viability compared to challenged controls. Protection against tbH\textsubscript{2}O\textsubscript{2} challenge was only seen when Nrf2 was first activated by a 24-hour treatment with tBHQ, causing complete normalization of ROS levels and viability (Figure 3B). This is seemingly in contrast with previous findings that did show protection against tbH\textsubscript{2}O\textsubscript{2} by adenoviral KLF2 overexpression alone, when assessed merely on the basis of morphology.\textsuperscript{25} We observed that the tbH\textsubscript{2}O\textsubscript{2}-challenged KLF2 cells indeed remained attached to their substratum, but also exhibit membrane blebbing and nuclear condensation, both signs of apoptosis (Figure 3C). It is likely that KLF2 overexpression sensitizes endothelial cells to tbH\textsubscript{2}O\textsubscript{2} exposure through the KLF2-mediated inhibition of transcriptional activity of NF-\kappa\textsuperscript{B}\textsuperscript{26} that confers endothelial protection against apoptosis by activating anti-apoptotic protein expression;\textsuperscript{27} as
well as expression of superoxide scavenging superoxide dismutases (SOD). In support of this hypothesis, manganese SOD (SOD2), which is an NFκB target rather than an Nrf2 target, was indeed found to be decreased by KLF2 overexpression.\(^5\)

In two recent reports, Nrf2-dependent gene expression was induced by atheroprotective levels of shear stress, which could be blocked by Nrf2 siRNA under flow.\(^10,11\) In the present study, a complementary approach was used with lentiviral overexpression of Nrf2. Next to previously described transcriptional targets like NQO1 and HO-1, several novel target genes were identified, including ESM1, MMP1, MMP3, SLC7A11, SRPX2, PLCG2 and HEPH. These genes indeed all have an ARE in conserved parts of their promoter suggesting they are direct targets of this transcription factor (Supplemental Table I). Of note, inspection of our array database suggested that these genes, with the exception of MMP1, were not upregulated by shear stress, indicating that under shear stress only a specific fraction of Nrf2-target genes is induced, implying combinatorial control involving other transcription factors. The obtained Nrf2-modulated transcriptome gave us the opportunity to perform a meta-analytic comparison between single genes and gene sets that are changed by shear stress, KLF2 and Nrf2 compared to their respective control conditions. Quite unexpectedly, our results show that a large part of the functional transcriptional network changes driven by shear stress coincide with the coordinated gene activation by the KLF2 and Nrf2 transcription factors (Figure 5). Still, these transcription factors govern largely independent transcriptomes and contribute different genes to the specific shear regulated gene sets and pathways.

Collectively our results imply a crucial, indirect role for KLF2 in attaining adequate protection against oxidative stress to endothelial cells. By increasing Nrf2 expression and nuclear translocation, KLF2 primes the Nrf2 pathway for activation, but subsequent activation of Nrf2 is needed for induction of antioxidant genes and phase II detoxifying enzymes. In conclusion, we postulate that KLF2 is an essential accessory factor for full activation of the antioxidant Nrf2 pathway by shear stress.
References


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