Laminar Flow Induction of Antioxidant Response Element-mediated Genes in Endothelial Cells

A NOVEL ANTI-INFLAMMATORY MECHANISM*

Xin Li Chen†‡, Signe E. Varner, Anjali S. Rao, Janice Y. Grey, Suzanne Thomas, Christopher K. Cook, Martin A. Wasserman, Russell M. Medford, Anil K. Jaiswal, and Charles Kunsch

From Discovery Research, AtheroGenics, Inc., Alpharetta, Georgia 30004 and Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77025

Atherosclerotic lesions preferentially develop in areas of the vasculature exposed to nonlaminar blood flow and low fluid shear stress, whereas laminar flow and high fluid shear stress are athero-protective. We have identified a set of genes including NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), ferroptin (heavy and light chains), microsomal epoxide hydrolase, glutathione S-transferase, and γ-glutamylcysteine synthase, whose expression is induced by exposure to prolonged physiological levels of steady laminar flow (shear stress = 20 dyn/cm²) in endothelial cells (EC). These genes contain an antioxidant response element (ARE) or ARE-like transcriptional regulatory sequence in their promoters and generally function to protect cells against oxidant stress. We demonstrate that exposure of EC to laminar flow activates ARE-mediated transcriptional activity. Mutation of the ARE from either the NQO1 or HO-1 promoter abolished laminar flow-induced NQO1 and HO-1 transcriptional activation. Expression of antisense Nrf2 (a transcriptional factor for ARE), a dominant negative Nrf2, or the cytoplasmic inhibitor of Nrf2 (Keap1/INrf2) inhibited laminar flow-induced NQO1 promoter activation in EC. In addition, expression of NQO1 or Nrf2 inhibited tumor necrosis factor-α-induced activation of VCAM-1 (vascular cell adhesion molecule-1) gene expression in EC. These data define the ARE as a novel endothelial shear stress response element. Furthermore, laminar flow activation of antioxidant genes via an ARE-dependent transcriptional mechanism may represent a novel athero-protective and anti-inflammatory mechanism in the vasculature.

Vascular endothelial cells are exposed to a tangential shearing force resulting from the flow of blood over the luminal surface of the vessel wall (1). The nature and magnitude of this fluid shear stress play a key role in the maintenance of vascular integrity and in the development of vascular diseases. For example, the nonrandom distribution of atherosclerotic lesions is due at least in part to local alterations in hemodynamic forces impinging on the vasculature (2–4). At sites vulnerable to lesion formation such as branch points, bifurcations, and curvatures, unidirectional laminar flow is disturbed, with areas characterized by complex flow patterns such as nonlaminar flow and flow reversal. In contrast, lesion-protected areas of the vasculature are characterized by more uniform laminar flow patterns with relatively high levels of fluid shear stress (2–4). It is now well accepted that areas of the vasculature exposed to nonlaminar or oscillatory flow have a predilection for the development of atherosclerotic lesions, whereas relatively high levels of steady laminar flow are athero-protective.

Differential regulation of endothelial gene expression by shear stress may be involved in the focal localization of early atherosclerotic lesions (5). For example, pro-atherogenic genes such as platelet-derived growth factor and monocyte chemotactrant protein-1 are transiently up-regulated by shear stress, peak at 1.5 h, and are followed by sustained down-regulation (6, 7). These initial responses of cultured endothelial cells to applied shear stress may reflect in vivo responses to the temporal and spatial changes of shear stress resulting from nonlaminar flow. Prolonged exposure of endothelial cells to oscillatory flow results in up-regulation of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1, and E-selectin (8), suggesting that oscillatory flow imparts a proinflammatory phenotype in endothelial cells. In contrast, physiological levels of laminar flow are anti-inflammatory and anti-adhesive. Prolonged exposure of murine endothelial cells to laminar flow results in down-regulation of the expression of VCAM-1, intercellular adhesion molecule-1, and E-selectin (9). We reported that chronic laminar flow suppresses interleukin-1β-induced VCAM-1 gene expression in endothelial cells (1). Similarly, exposure of endothelial cells to fluid flow inhibits monocyte adherence and VCAM-1 expression induced by TNF-α or oxidized low density lipoprotein in endothelial cells (10). In addition, genes encoding the antioxidants manganese superoxide dismutase, Cu,Zn superoxide dismutase, heme oxygenase-1 (HO-1), and endothelial nitric-oxide synthase are demonstrated to be up-regulated by laminar flow but not by oscillatory flow (11, 12).

The antioxidant response element (ARE), also referred to as the electrophile response element (EpRE), is a cis-acting regu-
latory element with a core sequence of 5′-RTGACNNNGC-3′ (13, 14). The ARE is present in the 5′-flanking regions of several genes encoding enzymes involved in the phase II metabolism of xenobiotics and antioxidant proteins including glutathione S-transferase (GST) (15), NADPH:quinone oxidoreductase (NQO1) (16), and glucuronosyltransferase (15). Activation of the ARE results from cellular exposure to insults that induce oxidative stress, including reactive oxygen species (ROS), ionizing radiation, and a variety of chemical entities including electrophilic compounds, lipid peroxides, antioxidants, Michael reaction acceptors, redox-cycling polyaromatic hydrocarbons, and quinones (13, 15). The ability of phase II enzymes to conjugate redox-cycling chemicals is an important protective mechanism against electrophile and oxidative toxicity (13). Although this antioxidant defense mechanism has been studied extensively as a hepatic detoxification mechanism, it has also been suggested that the ARE pathway may contribute to antioxidant defenses via induction of other ARE-regulated antioxidant proteins such as HO-1 (17) and ferritin (18). There is a considerable body of evidence that suggests that HO-1 plays an important protective role in the pathogenesis of a variety of diseases mediated via oxidant stress and may serve as a therapeutic target (19). For example, targeted expression of HO-1 prevents the pulmonary inflammatory and vascular responses to hypoxia (20). Induction of HO-1 in vascular cells suppresses oxidized low density lipoprotein-induced monocyt transmigration and inhibits atherosclerotic lesion formation in low density lipoprotein receptor knockout mice (21, 22). In addition, ferritin, an iron sequestant, is up-regulated in response to oxidative stress (18). Overexpression of ferritin protects endothelial cells from oxidant-mediated cytotoxicity (23).

Through the analysis of subtraction libraries, we have identified numerous genes whose expression is significantly increased by prolonged exposure to laminar flow as compared with static culture in human aortic endothelial cells (HAEC). In the present study, we report that exposure of endothelial cells to laminar flow activates ARE-driven transcriptional activity and increases the expression of a set of ARE-regulated genes. These genes include NQO1, HO-1, microsomal epoxide hydrolase, GST, ferritin, and γ-glutamylcysteine synthase. We further demonstrate that the ARE is the cis-acting element in the promoter of the NQO1 and HO-1 genes that mediates laminar flow-induced gene expression. Laminar flow-mediated, ARE-dependent NQO1 gene regulation in endothelial cells involves the transcriptional factor NF-E2-related factor 2 (Nrf2). Furthermore, co-expression of NQO1 or Nrf2 inhibited TNF-α-induced activation of VCAM-1 gene expression. These data suggest that laminar flow may exert athero-protective effects through a coordinated increase in expression of ARE-regulated intracellular antioxidant genes in endothelial cells. Therefore, this newly described laminar flow-regulated pathway may contribute to the modulation of oxidation-reduction (redox)-sensitive processes that result in vascular dysfunction, including the expression of inflammatory gene products.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HAEC were obtained from Clonetics, Inc. (San Diego, CA) and cultured in EGM-2 growth medium (Clonetics, Inc.). Cells were used between passages 5 and 9. Human microvascular endothelial cells (HMEC) were described previously (24) and were cultured in modified MCDB 131 (Invitrogen) supplemented with 10% fetal bovine serum and EGM Singlequots (Clonetics, Inc.). All cells were maintained at 37 °C under 5% CO2.

**Recombinant Plasmid**—To construct pXARE/Luc and pXmutARE/Luc, tandem repeats of double-stranded oligonucleotides spanning the ARE of the human NQO1 promoter, 5′-CATGCTAGCTGATCCGAA-GATC-3′ (for wild type ARE) and 5′-CATGCTAGCTGATCCGAA-GATC-3′ (for mutated ARE, the underlined sequences represent mutation of conserved nucleotides GC to TA in the ARE), were introduced into the SacI and BglII sites of pG3L promoter plasmid (Promega Corp.) (25).

pNQO1CAT1.55 contains coordinates −1550 to +110 of the human NQO1 gene cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (26). To generate a mutated ARE in the NQO1 promoter, pNQO1CAT1.55mARE, the conserved GC nucleotides in the ARE element (13, 18) were mutated to TA using GeneTailor mutagenesis kit according to manufacturer's instructions (Invitrogen). The PCR primers used in the mutagenesis were 5′-CGGAGTCGACGATGCTA-TAAAGAATTGAGCC-3′ (forward primer, the underlined TA represents GC to TA mutation) and 5′-TGAGTCGACGATGCTGCAA-GTTAAAAAGGC-3′ (reverse primer). To generate the human HO-1 promoter construct, pHO-Luc4.0mARE, a DNA fragment containing 4.0 kilobases of the human HO-1 promoter region was amplified from genomic DNA (Promega) using specific primers described previously (27) and cloned into the MluI and XhoI sites of pG3L basal plasmid (Promega). To generate a mutated ARE in the HO-1 promoter, pHO-Luc4.0mARE, the conserved GC nucleotides in the inverted ARE element were mutated to TA using the GeneTailor mutagenesis kit according to the manufacturer's instructions (Invitrogen). The PCR primers used in the mutagenesis were 5′-GGCGGATTTTGTCGATT-GATTGAATCGACGATGCTACCA-3′ (forward primer, the underlined GC represents GC to TA mutation) and 5′-TGGTCTTTCTCGACGATGCTACCAAAA-3′ (reverse primer).

LNXN-Nrf2 (an expression vector containing the full-length murine Nrf2 gene) and LNXN-Nrf2R (containing murine Nrf2 gene in an anti-sense orientation) have been described previously (28). To generate a dominant negative Nrf2 mutant, a DNA fragment that contains the Cap’nCollar homology region and the basic leucine-zipper domain (amino acids 399–598) was amplified by PCR reaction using the following primers: 5′-ATGGGCACGCTAGTGAAGGACCAGCTGCG-3′ (forward primer) and 5′-CTATGTTTATCTGCTAGTGT-3′ (reverse primer). The amplified fragment was cloned into the NheI and XhoI sites of pcDNA3 to generate pcDNA3NsnD-Nrf2. To construct NQO1 and Keap1/INrf2, p85VCAM/CAT is a chimeric reporter gene containing coordinates −85 to +12 of the human VCAM-1 promoter linked to the CAT gene (29).

**Flow System**—The flow system used has been previously described (8, 12). Briefly, HAEC or HMEC were seeded onto gelatin-coated glass slides and grown overnight before exposure to flow. Cells were exposed to either static conditions (cells maintained on glass slides in a 150-cm2 tissue culture dish) or exposed to flow conditions. The glass plate containing the monolayer was inserted into a parallel-plate flow chamber that was installed in a closed loop flow system. For laminar flow experiments, endothelial monolayers were subjected to laminar flow with shear stress of 0.5 dyn/cm2 or 2 dyn/cm2. For oscillatory flow experiments, the endothelial monolayers were exposed to low mean shear stress (less than 0.5 dyn/cm2) with a superimposed instantaneous oscillatory shear stress that cycled between +5 and −5 dyn/cm2 at a periodicity of 1 Hz. This small mean flow component was included to ensure adequate media exchange over the endothelial monolayer. After the indicated times, RNA or protein samples were collected.

**Subtraction Libraries**—Suppression subtraction hybridization libraries were prepared from total RNA collected from HAEC exposed to laminar flow or static cultures for 48 h using the Clontech PCR-Select(™) Subtraction kit (Clontech, Palo Alto, CA). This method for creating subtraction libraries is based on the method first described by von Stein et al. (30) and Mueller et al. (31). In general, this method “subtracts” low and high abundance mRNA populations that are in common between two RNA samples while at the same time equalizing and enriching for differentially expressed sequences. After construction of the subtracted libraries that were enriched for sequences preferentially in the laminar flow population or the static population, the PCR-Select Differential Screening kit (Clontech) was used to rapidly and efficiently amplify DNA from the libraries. Approximately 800 clones from each of the laminar flow-enriched and static-enriched libraries were hybridized to probes created from each library. Individual clones demonstrating a differential pattern of hybridization were selected for DNA sequence analysis. Clone identities were determined by BLAST analysis of the GenBank(™) DNA sequence data base.

**Gene Expression Analysis**—PCR oligonucleotide primers were designed to amplify an ~500-base pair fragment from the 3′ end of each mRNA (Table I). Total RNA was collected from HAEC using Trizol (Invitrogen). 3 μg of total RNA was reverse-transcribed into cDNA by reverse transcriptase (Invitrogen), and the level of each gene product
DNA sequences for primer pairs used in RT-PCR analysis

| Gene name                | GenBank™ Accession Number | Primer sequence       |
|--------------------------|--------------------------|-----------------------|
| γ-Glutamylcysteine synthase (heavy subunit) | M90656                   | F-TGAGGCAACTCACCAGGACGC<br>R-AAATCACTCCCCACAGGCAATT |
| Heme oxygenase-1         | XM_009946                | F-CTGCCGCTTTTCTCTACAG<br>R-CAGAGCTCTCACTCCCTCTCA |
| Microsomal epoxide hydrolase | NM_000120              | F-CTGGCCACTGACAAAAATGACC<br>R-GCTCCCCTCTTACCTAAATACT |
| Glutathione S-transferase| XM_012235                | F-GTTTTGCACTCCAGGAGAC<br>R-GCTCCCCTCTTACCTAAATACT |
| NQO1                     | J03934                   | F-CCTGGCCTTGAACCTTTATG<br>R-CAGCTGGCTCAACTCCCTCA |
| Ferritin (heavy chain)   | NM_002032                | F-CTGCCCACTGACAATGATTC<br>R-AAGCCGATCTAACTTGGAG |
| Ferritin (light chain)   | M10119                   | F-GCCCGAGGCGCTAGGAG<br>R-CAAATGCGAGGAGGAGGAG |
| β-Actin                  | XM_004814                | F-ATCAGGGCGCTCTACCT<br>R-GGGGCAAGAAGGCTCATC |

was measured by semi-quantitative end-point RT-PCR analysis. Cycling conditions were 95 °C initial denaturation for 5 min, 26 cycles of 94 °C denaturation (20 s), 55 °C annealing (30 s), and 72 °C extension (20 s), followed by a 7-min extension at 72 °C. After amplification, 15 μl of the sample was electrophoresed on a 1.5% agarose gel and stained by ethidium bromide. Relative band intensities were determined by densitometry using the Bio-Rad Quantity One software. For each primer pair, serial dilutions of each cDNA were assayed, and a dilution that resulted in linear amplification was used for further experiments. Each sample was assayed at least three times from the same RNA sample, and results are expressed as relative levels compared with β-actin.

Western Blot Analysis—HAEC were lysed for 30 min on ice in 1 ml of a lysis buffer containing 0.5% Nonidet P-40, 50 mM Hepes, pH 7.3, 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Protein samples (15 μg) were subjected to electrophoresis on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Antibody-bound protein bands were then visualized via horseradish peroxidase-dependent chemiluminescence (Amersham Biosciences). Rabbit anti-NQO1 antibody was described previously (32). Mouse monoclonal antibody against β-tubulin was purchased from Santa Cruz Biotechnology Inc. Relative band intensities were determined by densitometry using the Bio-Rad Quantity One software.

Transfection and Promoter Activity Assays—HMEC were grown to 70% confluence in 6-well plates and transfected with various plasmids as indicated in figure legends using SuperFect transfection reagents. 24 h after transfection, cells were lysed for 30 min on ice in 1 ml of a lysis buffer containing 0.5% Nonidet P-40, 50 mM Hepes, pH 7.3, 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Protein samples (15 μg) were subjected to electrophoresis on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane. Antibody-bound protein bands were then visualized via horseradish peroxidase-dependent chemiluminescence (Amersham Biosciences). Rabbit anti-NQO1 antibody was described previously (32). Mouse monoclonal antibody against β-tubulin was purchased from Santa Cruz Biotechnology Inc. Relative band intensities were determined by densitometry using the Bio-Rad Quantity One software.

RESULTS

Laminar Flow Increases the Expression of ARE-containing Genes—To identify genes that were differentially expressed by exposure to laminar flow, we constructed cDNA subtraction libraries from HAEC kept in static culture or subjected to laminar flow (shear stress = 20 dyn/cm²) for 48 h. Differentially expressed clones from each enriched library were identified by differential hybridization, and several hundred clones from each library were selected for DNA sequencing. DNA sequence analysis identified many genes that were relatively abundant in the laminar flow-enriched library compared with the static-enriched library. Analysis of the promoters of a subset of these genes including ferritin (heavy and light chains), NQO1, HO-1, GST, microsomal epoxide hydrolase, and γ-glutamylcysteine synthase revealed a common transcriptional regulatory feature. Each of these genes contains an ARE or ARE-like transcriptional regulatory element(s) in its promoter and is known to be up-regulated by oxidative stress. Furthermore, these genes function as antioxidants and protect cells from oxidative stress. Table II shows the sequence alignment of the ARE or ARE-like regulatory elements from the laminar flow-inducible genes compared with the consensus ARE sequence, RTGACNNGGC (13, 14). These observations provide the first suggestion that the AREs may mediate laminar flow-induced gene expression in endothelial cells.

To confirm our results from the subtraction libraries that laminar flow induces the expression of these genes, mRNA levels were further evaluated by semi-quantitative RT-PCR. As shown in Fig. 1, all of the genes were induced, although to varying degrees, by exposure to laminar flow for 48 h. These observations confirm that exposure of HAEC to laminar flow for 48 h results in increased expression of ARE-containing cytoprotective genes compared with static controls.

To characterize the role of fluid flow in the regulation of ARE-mediated gene expression, we focused on the NQO1 and HO-1 genes. The effects of time, flow rates, and types of flow were evaluated for their contribution to NQO1 gene expression. As shown in Fig. 2A, the expression of NQO1 in response to laminar flow increased progressively from 12 to 48 h. Similarly, the expression of HO-1 in response to laminar flow increased progressively from 6 to 48 h (Fig. 2B). In contrast, the levels of NQO1 and HO-1 mRNA in static cultures remained relatively constant throughout the time course (Fig. 2). As shown in Fig. 3, the expression of NQO1 in response to laminar flow at 48 h was also dependent on the levels of shear stress. As compared with static cultures, laminar flow with shear stress of 5 dyn/cm² increased the NQO1 mRNA levels ~7-fold. Increasing the shear stress to 20 dyn/cm² resulted in an ~14-fold increase in NQO1 mRNA levels at 48 h. These data demonstrate that higher levels of shear stress result in greater expression of NQO1 in endothelial cells.

Several endothelial cell genes are differentially regulated in response to either laminar or nonlaminar flow. Laminar flow is believed to confer an anti-inflammatory, anti-oxidant phenotype to the vasculature, whereas nonlaminar flow or oscillatory flow imparts a greater oxidant stress and a more pro-inflammatory phenotype. We compared the effects of oscillatory flow and laminar flow on NQO1 gene expression at 48 h. As shown in Fig. 3, although exposure of HAEC for 48 h to oscillatory flow with shear stress of ~5 dyn/cm² induced approximately a 3-fold increase in NQO1 mRNA levels, the magnitude of increase was smaller compared with the more than 7-fold increase in HAEC treated with laminar flow of the same magnitude of shear stress (5 dyn/cm²). These results suggest that not only the flow pattern (laminar versus oscillatory) but also the absolute level
of shear stress differentially regulates NQO1 gene expression in endothelial cells.

We further examined the effects of laminar or oscillatory flow on NQO1 protein levels by Western blot analysis. As shown in Fig. 4, exposure of HAEC for 48 h to oscillatory flow (shear stress 5 dyn/cm²) resulted in a 2-fold increase in NQO1 protein level compared with static culture. Exposure of HAEC to laminar flow (shear stress 20 dyn/cm²) induced approximately a 6-fold increase in NQO1 protein compared with static culture. These data confirm the results observed with NQO1 mRNA levels (Fig. 3B), demonstrating that physiological levels of laminar flow are a potent activator of ARE-regulated genes such as NQO1.

Laminar Flow Activates ARE-regulated Genes

Because laminar flow induces the expression of a set of genes that contain the ARE regulatory element, we hypothesized that the ARE or ARE-like sequences may represent a novel and important cis-acting element that confers inducible expression in response to shear stress. To confirm that the ARE functions as a shear stress-responsive transcriptional control element, we transiently transfected HMEC with p3xARE/Luc and subjected cells to laminar flow (shear stress 20 dyn/cm²) for 24 h. HMEC were used because HAEC are relatively refractory to transient transfection. Similar to HAEC, laminar flow induced the expression of ARE-regulated genes in HMEC (data not shown). As shown in Fig. 5A, laminar flow treatment of HMEC resulted in a dramatic increase in the ARE-driven promoter activity. In contrast, mutation of the conserved GC nucleotides to TA abolished laminar flow-induced ARE-driven promoter activity. These data demonstrate that the ARE is a newly identified shear stress response element in endothelial cells.

To further determine the role of the ARE in laminar flow-induced gene expression, we used transient transfection assays with the wild type and ARE mutated NQO1 or HO-1 promoters. The data shown in Fig. 5B demonstrate that exposure of HMEC for 24 h to laminar flow resulted in substantial activation of the wild type NQO1 promoter. In contrast, when HMEC were transfected with pNQO1CAT1.55mARE (containing point mutations of the ARE in the NQO1 promoter), no activation was observed by laminar flow (Fig. 5B). Similarly, exposure of

| Genes                     | Sequences                        | References |
|---------------------------|----------------------------------|------------|
| Human NQO1                | gcagtcaca GTGAC tca GC agaatct   | 25         |
| Murine ferritin (heavy chain) | cctcc GTGAC tca GC agaatct   | 25         |
| Murine ferritin (light chain) | tcagc GTGAC tca GC agaac     | 18         |
| Human HO-1                | GTGAC tca GC a                  | 62         |
| Human HO-1                | ATGAC tca GC a                  | 62         |
| Murine GST                | gctaag GTGAC aaa GC aaacctt     | 15         |
| Human γ-glutamylcysteine  | cagctcccc GTGAC tca GC gcttt    | 63         |
| Consensus                 | nnnn ATGAC nnn GC nnn           | 13,14      |

FIG. 1. Effects of laminar flow on the expression of ARE-mediated genes in endothelial cells. The genes examined include ferritin (heavy (H) and light (L) chains), quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), microsomal epoxide hydrolase (mEH), GST, and γ-glutamylcysteine synthase (γ-GCS). HAEC grown on glass plates were kept in static conditions or subjected to laminar flow with shear stress at 20 dyn/cm² (LF) for 48 h. Relative mRNA levels were determined by semi-quantitative RT-PCR and normalized to β-actin levels. Values are the means ± S.D., n = 3, *p < 0.05 compared with static culture.

FIG. 2. Temporal effects of laminar flow on NQO1 and HO-1 mRNA levels in endothelial cells. HAEC were kept in static culture or subjected to laminar flow with shear stress at 20 dyn/cm² (LF) for the indicated times. Relative NQO1 (A) and HO-1 (B) mRNA levels were determined by semi-quantitative RT-PCR and normalized to β-actin levels. Values are the means ± S.D., n = 3, *p < 0.05 compared with static culture.
oscillatory flow (\(H_11006\) p*, \(tibodies against NQO1 or were subjected to electrophoresis and Western blot analysis using antibodies against NQO1 or \(\beta\)-tubulin. Two independent experiments showed similar results.

Laminar Flow Activates ARE-regulated Genes

HMEC for 48 h to laminar flow activated wild type HO-1 promoter pHO-1Luc4.0 (Fig. 5C). However, mutation of the ARE in the HO-1 promoter abolished laminar flow-induced ARE-driven HO-1 promoter activity (Fig. 5C). These observations directly demonstrate that laminar flow activation of the NQO1 and HO-1 genes in endothelial cells occurs at the transcriptional level. More importantly, activation of NQO1 and HO-1 by laminar flow requires a functional ARE element. Cumulatively, these data indicate that the ARE transcriptional element is an important mediator of signals generated in endothelial cells by exposure to shear stress.

**Nrf2 Mediates Laminar Flow-induced Expression of NQO1 in Endothelial Cells**—Nrf2 is a Cap’n’Collar and leucine zipper-containing transcriptional factor and plays a critical role in ARE-mediated gene expression (34, 35). However, the role of Nrf2 in endothelial cell gene expression is unknown. To investigate whether Nrf2 can activates the ARE-driven promoter in endothelial cells, p3xARE/Luc was transfected with an expression vector encoding Nrf2 into HMEC. As shown in Fig. 6A, co-expression of Nrf2 in HMEC activated the ARE-driven promoter activity. When Nrf2 was co-transfected with the p3xmutARE/Luc, a mutated ARE version of the ARE-driven promoter, no activation was observed by Nrf2. To examine whether Nrf2 can activate NQO1 transcription through an ARE-dependent mechanism in endothelial cells, pNQO1CAT1.55 or ARE mutant pNQO1CAT1.55mARE was co-transfected along with an expression vector encoding Nrf2 into HMEC. As shown in Fig. 6B, co-expression of Nrf2 in HMEC activated wild type NQO1, but not ARE mutant NQO1, gene transcription from the reporter construct. These data are consistent with the known role of Nrf2 in ARE-dependent activation of NQO1 in other cell types (28) and demonstrate for the first time a role of Nrf2 in ARE-mediated transcriptional activation in endothelial cells.

To examine whether Nrf2 mediates laminar flow-induced activation of NQO1 in endothelial cells, pNQO1CAT1.55 was co-transfected with LNCX-Nrf2R (an expression vector encoding antisense Nrf2) or a dominant negative Nrf2 mutant pcdNA3DN-Nrf2 into HMEC. As shown in Fig. 7A, co-transfection with antisense Nrf2 suppressed laminar flow-induced activation of NQO1 gene expression. Similarly, co-transfection with dominant negative Nrf2 suppressed both basal and laminar flow-induced activation of Nrf2 gene transcription (Fig. 7B).
Keap1/INrf2 Inhibits Laminar Flow-induced Activation of NQO1 Gene Expression—Recent studies suggest that Nrf2 activity is normally repressed through its localization in the cytoplasm by binding to an inhibitor protein called Keap1 (36). The rat homologue of Keap1 was recently cloned and named INrf2 (37). We will refer to this protein as Keap1/INrf2. It has been suggested that the Nrf2-Keap1/INrf2 interaction may constitute a cytoplasmic sensor for oxidative stress (36). Electrophilic agents release Nrf2 from its complex with Keap1/INrf2, allowing the translocation of Nrf2 from the cytoplasm to the nucleus to activate transcription (36, 37). To further examine the role of Nrf2 and to determine whether Keap1/INrf2 plays a role in regulating laminar flow-induced ARE-mediated transcriptional activation in endothelial cells, we evaluated laminar flow-inducible activation of the NQO1 gene in the presence and absence of Keap1/INrf2. As shown in Fig. 7C, overexpression of Keap1/INrf2 in HMEC inhibited both basal and laminar flow-mediated activation of NQO1 promoter activity. However, in the presence of Keap1/INrf2, laminar flow was still able to increase NQO1 gene transcription although to a lesser degree than vector only transfected cells. These data suggest that under these experimental conditions there was perhaps an insufficient sequestration of Nrf2 by Keap1/INrf2 under the laminar flow condition. Cumulatively, these data demonstrate that the Nrf2-Keap1/INrf2 pathway regulates ARE-dependent transcription, and this pathway is involved in regulation of laminar flow-induced ARE transcriptional activation in endothelial cells.
Laminar Flow Activates ARE-regulated Genes

These cells were also transfected with 0.5 μg of p35VCAM-1/CAT plus 1 μg of pCDNA3-NQO1 (NQO1), LNCX-Nrf2 (Nrf2) or pCDNA3-LacZ (Vector). These cells were also transfected with 0.5 μg of pRL-TK for normalization of transfection efficiency. After a 24-h recovery, cells were treated with or without TNF-α for 16 h, cell extracts were harvested, and 5 μg of protein was used for CAT assays. Values are means ± S.D., n = 4, *p < 0.05 compared with control group.

NQO1 and Nrf2 Modulate TNF-α-mediated VCAM-1 Gene Expression—Cytokine-activated VCAM-1 gene expression is regulated by an oxidation-reduction (redox)-sensitive mechanism in endothelial cells (29). We hypothesized that the coordinate induction of ARE-containing antioxidant genes through activation of Nrf2 may be one mechanism whereby fluid flow may modulate redox-sensitive gene expression in endothelial cells. Because TNF-α-inducible expression of the VCAM-1 promoter is sensitive to redox modulation (29), we used the VCAM-1 promoter and transient transfection in HMEC as a readout for expression of a redox-sensitive inflammatory gene. We sought to determine whether activation of the ARE pathway via overexpression of Nrf2 or expression of NQO1 would modulate cytokine-inducible VCAM-1 gene expression. As shown in Fig. 8, TNF-α induced a significant increase in VCAM-1 promoter activity. Expression of either NQO1 or Nrf2 in HMEC dramatically inhibited TNF-α-induced activation of the VCAM-1 promoter. These observations provide support for the notion that activation of ARE-containing genes may modulate redox-sensitive inflammatory gene expression in endothelial cells.

DISCUSSION

Many studies suggest that laminar flow may protect the vasculature from early atherosclerotic lesions; however, the underlying mechanisms are still unclear. In the present study, we used an in vitro model of HAEC exposed to physiological levels of laminar flow (shear stress = 20 dyn/cm²), which approximate the average wall shear stress typically encountered in lesion-protected areas of the vasculature. In addition, we have chosen to examine the biological effects of fluid flow in our model system at 48 h, because we believe that exposure to shear stress for relatively long periods are more relevant to the in vivo flow conditions. We found that the expression of a set of cytoprotective and antioxidant genes was coordinately induced in endothelial cells upon exposure to laminar flow. These genes contain ARE or ARE-like transcriptional regulatory element(s) in their promoters. For the first time, we demonstrated that the ARE represents a new transcriptional element responsive to shear stress in endothelial cells. Functional experiments demonstrate that activation of ARE-driven genes through the transcriptional factor Nrf2 or via overexpression of one of these cytoprotective genes, NQO1, represses the TNF-α-induced expression of the redox-sensitive adhesion molecule, VCAM-1, in endothelial cells. These experiments provide a novel molecular mechanism that help explain the athero-protective and antioxidant nature of laminar flow. Activation of the ARE and ARE-regulated anti-oxidant and cytoprotective genes by laminar flow likely contributes to defensive mechanisms to protect against oxidant-mediated endothelial dysfunction and redox-sensitive inflammatory gene expression.

Previous studies showed that fluid shear stress regulates endothelial gene expression through various cis-elements including the 12-O-tetradecanoylphorbol-13-acetate-responsive element (38), the stress response element (39), NF-κB (40–42), and an early growth response-1 recognition element (40, 43). For example, the shear stress response element is found in the promoter region of platelet-derived growth factor-B chain and intercellular adhesion molecule-1 gene (39, 44), and the early growth response-1 binding site is required for the activation of platelet-derived growth factor-A chain gene by shear stress (40, 43). Platelet-derived growth factor is a potent mitogen and chemoattractant for smooth muscle cells. Activation of 12-O-tetradecanoylphorbol-13-acetate-responsive element is responsible for shear stress-induced monocyte chemoattractant protein-1, a potent chemoattractant for monocytes (38). NF-κB is involved in the up-regulation of a variety of immune and inflammatory genes including VCAM-1 (29, 45, 46). It is notable that these shear stress-regulated cis elements are commonly found in the promoters of pro-atherogenic or pro-inflammatory genes.

In contrast, the ARE represents a new shear stress response element that coordinately regulates cytoprotective and athero-protective genes in endothelial cells. These proteins, such as GST, NQO1, γ-glutamylcysteine synthase, HO-1, and the light and heavy chains of ferritin share a common role as antioxidants (13, 23). HO-1 catalyzes the rate-limiting reaction in heme degradation to CO and biliverdin, a potent antioxidant (47). CO may influence vessel tone, mitogenesis, and inflammatory responses in the vasculature (48). Recently, Hayashi et al. (49) demonstrated that HO-1 attenuates leukocyte-endothelial cell adhesion in vivo through the action of bilirubin, a product from biliverdin (49). Overexpression of HO-1 has been shown to inhibit atherosclerotic lesion formation in low density lipoprotein receptor knock-out mice and in Watanabe heritable hyperlipidemic rabbits (22, 50). Ferritin is another cytoprotective protein. Ferritin binds free iron and prevents it from participating in iron-catalyzed free radical reactions and the generation of oxidative stress. The regulation of ferritin gene expression in response to oxidative stress is mediated via two AREs in the ferritin promoter (18). GST catalyzes the S-conjugation of glutathione with reactive species such as electrophilic compounds (51). γ-Glutamylcysteine synthase catalyzes the rate-limiting reaction in the synthesis of glutathione, which is an efficient ROS scavenger (52). Because ROS contribute to the pathogenesis of atherosclerosis by stimulating inflammatory gene expression and promoting smooth muscle cell proliferation, activation of ARE-regulated antioxidant proteins and enzymes may help to counterbalance the biological effects of ROS and to maintain intracellular redox homeostasis. Therefore, shear stress-mediated activation of this antioxidant defense pathway via the ARE defines a new athero-protective mechanism in the vascular endothelium. Conversely, extrapolation of our in vitro findings suggest that decreased levels of these ARE-regulated cytoprotective genes may be present in vivo at areas of low shear stress, which would be more predisposed to oxidative damage and the activation of redox-sensitive inflammatory signals.

The present study demonstrates that Nrf2 is required for laminar flow-induced NQO1 gene expression in endothelial cells. Nrf2 plays a critical role in ARE-mediated gene expres-
Laminar Flow Activates ARE-regulated Genes

...ion and in the regulation of biological responses to oxidative stress. For example, cells from Nrf2 knockout mice are deficient in their ability to induce anti-oxidative stress genes and as a result are highly sensitive to oxidative stress-induced cell death (34, 35). In Nrf2-deficient macrophages, a number of anti-oxidative stress genes could no longer be induced by electrophilic or ROS-generating agents (53). Nrf2 is normally maintained in the cytoplasm through its interaction with Keap1/INrf2 (36, 37). Upon stimulation by oxidative stress, Nrf2 is believed to dissociate from Keap1/INrf2 and translocate to the nucleus where it binds to the ARE (36).

It has been suggested that the Nrf2-Keap1/INrf2 interaction may constitute a cytoplastic sensor for oxidative stress (54). Exposure of endothelial cells to fluid flow stimulates the generation of ROS through the activation of NADPH oxidase (55, 56). It is possible that the induction of ARE-regulated genes in response to laminar flow is a compensatory response to elevated levels of ROS. De Keulenaer et al. (12) demonstrate that the flow patterns differentially modulate endothelial cell redox state. Steady laminar flow induced a transient increase in superoxide production that returned to baseline after 24 h, whereas oscillatory flow induced a higher and sustained increase in superoxide production. However, we observe a continual increase in ARE gene expression up to 48 h after exposure to laminar flow, suggesting that it is not solely the “burst” of ROS after exposure to laminar flow that induces ARE expression. The present study shows that laminar flow is more potent in the activation of ARE-mediated gene expression compared with oscillatory flow. In addition, the magnitude of fluid flow-induced expression of the ARE-mediated NQO1 gene is dependent on the levels of shear stress. A high level of laminar flow (shear stress = 50 dyn/cm²) induces significantly higher expression of ARE-mediated genes than a low level of laminar flow (shear stress = 5 dyn/cm²) and oscillatory flow (shear stress = 5 ± 5 dyn/cm²). These data suggest that laminar flow may use specific mechanoreceptor(s) to activate the Nrf2/ARE pathway; however, additional experiments are needed to determine if laminar flow-mediated ARE activation occurs directly through biomechanical stimulation or indirectly via modulation of ROS.

It is well accepted that redox-sensitive regulation of inflammatory gene expression in vascular cells contributes to the pathogenesis of atherosclerosis (57). ROS such as H₂O₂ stimulate monocyte chemoattractant protein-1 gene expression in vascular smooth muscle cells (58). Inflammatory stimuli-activated endothelial expression of monocyte chemoattractant protein-1 and VCAM-1 is inhibited by antioxidants such as pyrrolidine dithiocarbamate (29, 46). Furthermore, inhibition of superoxide generation in endothelial cells suppressed cytokine-induced VCAM-1 gene expression (59). These studies suggest that increasing intracellular antioxidant capacity may be therapeutically beneficial for chronic inflammatory diseases such as atherosclerosis by controlling redox-sensitive vascular gene expression. In this report, we demonstrate that co-expression of Nrf2 or NQO1 can suppress cytokine-induced VCAM-1 gene expression. Presumably, overexpression of Nrf2 coordinately induces expression of multiple ARE-containing genes in endothelial cells and functions to reduce cytokine-induced oxidant signals that modulate redox-sensitive gene expression such as VCAM-1. Our data suggest that increased expression of the ARE-mediated genes has a potential athero-protective effect and is anti-inflammatory in endothelial cells. These data suggest that laminar shear stress induces a protective phenotype to the cells in part by activating the expression of genes that function to protect endothelial cells from oxidative stress. These data further confirm the notion that physiological levels of shear stress are essentially anti-inflammatory and athero-protective.

NQO1 (and its homologue NQO2) are ubiquitous flavoproteins found in eukaryotes. NQO1 catalyzes the two electron reductive metabolism of toxic quinones and their derivatives and converts them to hydroxyl quinones for elimination (60). The obligatory two-electron reduction of quinones catalyzed by NQO1 competes with the one-electron reduction by other deoxidation enzymes that generate unstable semiquinones that undergo redox cycling in the presence of molecular oxygen, leading to the formation of reactive oxygen species (16). Another major function of NQO1 is to maintain coenzyme Q (ubiquinol) in the antioxidant form (ubiquinone) in membranes (60). Coenzyme Q is a lipid-soluble constituent of membranes and possesses “membrane-stabilizing” activity and inhibition of membrane lipid peroxidation. Ubiquinols can react with oxygen radicals and, thus, prevent direct damage to biomolecules and initiation of lipid peroxidation (61). Thus, NQO1 has the potential to protect against oxidative stress and lipid peroxidation in the vascular wall. We show that overexpression of NQO1 inhibits the TNF-α-induced expression of VCAM-1. These data support the notion that NQO1 may protect against cytokine-induced oxidative stress and may contribute to the regulation of expression of redox-sensitive inflammatory genes in the vasculature.

The discovery that the ARE represents a unique transcriptional element in response to physiologically athero-protective laminar flow in endothelial cells extends our understanding of the role of this important transcriptional regulatory network and the importance of ARE-regulated genes in vascular biology and diseases. Increased expression of antioxidant and cytoprotective genes via activation of the ARE likely contributes to the athero-protective and anti-inflammatory effects of laminar flow. Modulation of endothelial ARE activity may represent a new approach for the prevention and treatment of atherosclerosis and other inflammatory diseases.

REFERENCES

1. Nirem, R. M., Alexander, R. W., Chappell, D. C., Medford, R. M., Varner, S. E., and Taylor, W. R. (1998) Am. J. Physiol. 316, 169–175
2. Asakura, T., and Kario, T. (1990) Circ. Res. 66, 1045–1056
3. Cornhill, J. F., Akins, D., Hutson, M., and Chandler, A. B. (1980) Atherosclerosis 33, 77–86
4. Montenegro, M. R., and Eggen, D. A. (1968) Lab. Invest. 18, 586–593
5. Resnick, N., and Ginibre, M. A., Jr. (1995) FASEB J. 9, 874–882
6. Shyy, Y. J., Heisch, H. J., Usami, S., and Chien, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4676–4682
7. Heisch, H. J., Li, N. Q., and Francos, J. A. (1991) Am. J. Physiol. 260, H642–H646
8. Chappell, D. C., Varner, S. E., Nirem, R. M., Medford, R. M., and Alexander, R. W. (1988) Circ. Res. 62, 532–539
9. Korenaga, R., Ando, J., Kosaki, K., Ishikii, M., Takada, Y., and Kamiya, A. (1997) Am. J. Physiol. 273, C1506–C1515
10. Tao, P. S., Buttrago, R., Chan, J. R., and Cooke, J. P. (1996) Circulation 94, 1682–1689
11. Topper, J. N., Cui, J., Falb, D., and Ginibre, M. A., Jr. (1996) Proc Natl. Acad. Sci. U. S. A. 93, 10417–10422
12. De Keulenaer, G. W., Chappell, D. C., Ishizaka, N., Nirem, R. M., Alexander, R. W., and Griendling, K. R. (1998) Circ. Res. 82, 1094–1101
13. Jaiswal, A. K. (1994) Biochem. Pharmacol. 48, 439–444
14. Wasserman, W. M., and Fahl, W. E. (1997) Proc Natl. Acad. Sci. U. S. A. 94, 5361–5366
15. Prestera, T., and Talalay, P. (1995) Proc Natl. Acad. Sci. U. S. A. 92, 8965–8969
16. Jaiswal, A. K. (2000) Free Radic. Biol. Med. 29, 254–262
17. Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999) J. Biol. Chem. 274, 29071–29078
18. Tsuji, Y., Ayaki, H., Whitman, S. P., Morrow, C. S., Torti, S. V., and Torti, F. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8798–8803
19. Immensheu, S., and Ramadarei, G. (2000) Biochem. Pharmacol. 60, 1121–1128
20. Minamino, T., Christou, H., Heisch, C. M., Liu, Y., Dhawan, V., Abraham, N. G., Perrellia, M. A., Mitsialis, S. A., and Kourambanas, S. (2001) Proc Natl. Acad. Sci. U. S. A. 98, 8789–8803
21. Ishikawa, K., Nakab, M., Leitinger, N., Fogelman, A. M., and Lasius, A. J. (1997) J. Clin. Invest. 100, 1209–1216
22. Ishikawa, K., Sugawara, D., Ooto, J., Watanabe, Y., Kawamura, K., Shiomi, M., Kabe, H., and Maruyama, Y. (2001) Circulation 104, 1831–1836
23. Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J. W., and Verrasolli, G. M. (1992) J. Biol. Chem. 267, 18148–18153
Laminar Flow Induction of Antioxidant Response Element-mediated Genes in Endothelial Cells: A NOVEL ANTI-INFLAMMATORY MECHANISM

Xi-Lin Chen, Signe E. Varner, Anjali S. Rao, Janice Y. Grey, Suzanne Thomas, Christopher K. Cook, Martin A. Wasserman, Russell M. Medford, Anil K. Jaiswal and Charles Kunsch

J. Biol. Chem. 2003, 278:703-711.
doi: 10.1074/jbc.M203161200 originally published online October 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203161200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 62 references, 33 of which can be accessed free at http://www.jbc.org/content/278/2/703.full.html#ref-list-1