Anoxia/Reoxygenation-Induced Tolerance With Respect to Polymorphonuclear Leukocyte Adhesion to Cultured Endothelial Cells

A Nuclear Factor-κB–Mediated Phenomenon

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Abstract—Exposing human umbilical vein endothelial cells (HUVECs) to anoxia/reoxygenation (A/R) results in an increase in polymorphonuclear leukocyte (PMN) adhesion to HUVECs. This A/R-induced hyperadhesion is completely prevented by a previous (24 hours earlier) exposure of HUVECs to A/R. This phenomenon has been termed “A/R tolerance.” Exposing HUVECs to A/R induces an increase in nuclear factor κB (NF-κB) in HUVEC nuclei within 4 hours. Interfering with either NF-κB activation (proteasome inhibitor) or translocation (double-stranded oligonucleotides containing NF-κB binding sequence) prevents the development of A/R tolerance (ie, the increase in A/R-induced PMN adhesion to HUVECs is the same after the first and second A/R challenges). NO production by HUVECs is increased after the second A/R challenge, but not after the first A/R challenge. Inhibition of NO synthase (NOS) during the second A/R challenge prevents the development of A/R tolerance with respect to PMN adhesion. However, while HUVECs contained endothelial NOS protein, no inducible NOS was detected in either tolerant or nontolerant cells. Further studies indicated that inhibition of GTP-cyclohydrolase I (an enzyme involved in de novo synthesis of an important cofactor for NOS activity, tetrahydrobiopterin) prevented the generation of NO in A/R-tolerant cells. Extracellular generation of NO (NO donor) did not effect the hyperadhesion response induced by the initial A/R challenge. A/R also induced an oxidant stress in naive HUVECs, but not in A/R-tolerant HUVECs. Inhibition of NOS during the second A/R insult results in the generation of an oxidant stress similar to that observed after the first A/R challenge. Taken together, the findings of the present study are consistent with a role for NF-κB in the development of A/R tolerance (with respect to PMN adhesion), perhaps by transcriptional regulation of GTP-cyclohydrolase. The increased NO production during the second A/R insult reduces PMN adhesion most likely by reducing the intracellular oxidant stress induced by A/R. (Circ Res. 1999;84:103-112.)

Key Words: nitric oxide • nitric oxide synthase • GTP-cyclohydrolase I • tetrahydrobiopterin

Reperfusion of previously ischemic tissues results in a series of events reminiscent of an acute inflammatory response.1,2 Intravital microscopy approaches have shown that the microvasculature, particularly the postcapillary venules, is the initial target of ischemia/reperfusion (I/R)–induced dysfunction. I/R results in an increase in leukocyte adhesion to postcapillary venules and emigration into the interstitium, as well as an increase in the microvascular permeability to albumin. Antioxidants, antagonists of lipid mediators (platelet-activating factor [PAF] and leukotriene B4 [LTB4]), and immunoneutralization of adhesion molecules on neutrophils (CD11/CD18) or endothelial cells (intercellular adhesion molecule-1) have all been shown to reduce both the neutrophil-endothelial cell adhesion interactions and the increase in vascular permeability noted after I/R.

In vitro models have been developed to mimic the microvascular dysfunction elicited by I/R. This approach involves exposing cultured endothelial cells to anoxia (or hypoxia) and subsequently reoxygenating them (anoxia/reoxygenation; A/R). Challenging endothelial cells with A/R results in increases in (1) oxidant production, (2) activation of nuclear transcription factors (nuclear factor κB [NF-κB] and activator protein-1), (3) adhesion molecule expression, (4) increased adhesivity to neutrophils, and (5) permeability to macromolecules.1 These in vitro approaches have allowed for mechanistic studies not readily performed using in vivo models. For example, supernatants obtained from A/R-conditioned endothelial cells can increase surface expression of CD11/CD18 on neutrophils and promote neutrophil adhesion to naive endothelial monolayers, an effect inhibited by antioxidants and a PAF receptor antagonist.3 In addition, in vitro approaches have allowed for the characterization of the contribution of various adhesion molecules on endothelial cells and neutrophils at different times after reoxygenation.4
together, the in vitro model of A/R very closely simulates the situation in vivo after I/R and allows for a more in-depth analysis of the cellular and molecular events involved in this pathology.

Although I/R induces a leukocyte-mediated microvascular and parenchymal cell dysfunction in affected tissue, I/R can also induce a series of events that renders the tissue more resistant to a subsequent I/R insult. This phenomenon, termed “I/R tolerance” has been demonstrated in the heart, brain, and intestine. Typically, I/R tolerance requires several hours or days to develop, indicating that some transcriptional event may be involved. Studies in the intestine indicate that the development of I/R tolerance is not due to an adaptational response of the parenchymal cells but to some cell type in the lamina propria. Endothelial cells are a resident cell population of the lamina propria of a variety of organ systems. They can also mount an adaptational response to an oxidant stress that renders them less susceptible to the injurious effects of a second oxidant challenge imposed several hours later. Thus, to study the mechanisms involved in the development of I/R tolerance, we focused our attention on endothelial cells and used our in vitro model of A/R to define some of the key cellular events involved in this phenomenon.

The present study is the first to demonstrate that endothelial cells can, indeed, develop A/R tolerance with respect to neutrophil adhesion to endothelium (ie, the typical A/R-induced increase in neutrophil adhesion is abolished if the endothelial cells are pretreated with an A/R insult). We also present evidence indicating that activation and translocation of the nuclear transcription factor (NF-kB) to HUVEC nuclei plays a critical role in the development of this tolerance. Furthermore, we have dissected out some of the critical pathways involved in the NF-kB-mediated development of A/R tolerance in endothelial cells and propose a working hypothesis for future studies of this phenomenon.

Materials and Methods

Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were harvested from umbilical cords by collagenase treatment (Worthington Biochemical, Inc, Freehold, NJ) as previously described. The cells were grown in medium M199 (GIBCO) supplemented with 10% heat-inactivated FCS (Intergen, Purchase, NY), thymidine (2.4 mg/mL), heparin sodium (10 IU/mL), penicillin (100 IU/mL), streptomycin (100 μg/mL) (Sigma Chemical Co), and endothelial cell mitogen (80 μg/mL) (Biomedical Technologies, Stoughton, Mass). The cell cultures were incubated at room air with 5% CO₂, 37°C, and 95% humidity and expanded by brief trypsinization with 0.25% trypsin in PBS containing 0.025% EDTA. First through third–passage HUVECs were seeded into fibronectin-coated 48-well tissue culture plates (Costar) and used for experiments when confluent.

Neutrophils

Human neutrophil polymorphonuclear leukocytes (PMNs) were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on Histopaque-1077 (Sigma Chemical Co). This procedure yields a PMN population that is 95% to 98% viable (trypan blue exclusion) and 98% pure (acetic acid/crystal violet staining).

A/R Protocol

The in vitro model of I/R used in the present study is similar to that described previously. Briefly, confluent HUVECs monolayers were exposed to anoxia by incubation in a Plexiglas chamber that was continuously purged (1 L/min) with an anoxic gas mixture (93% N₂, 5% CO₂, and 2% H₂). To ensure an oxygen-free environment, the gas mixture was passed through a catalytic deoxygenator (Fisher Chemical) before entry into the chamber. Temperature in the chamber was maintained at 37°C by a heating pad. After a 30-minute period of anoxia, reoxygenation was initiated by exposing the endothelial cells to room air in a CO₂-cell culture incubator (A/R). As a control, HUVECs were identically treated except that they were exposed to normoxia (21% O₂, 5% CO₂, and 74% N₂) instead of anoxia (normoxic controls; normoxia/reoxygenation, N/R).

PMN Adhesion Assay

Isolated neutrophils were suspended in PBS buffer and radiolabeled by incubating the cells at 5 × 10⁸ cells/mL with a 50 μCi Na¹³¹I/mL PMN suspension at 37°C for 60 minutes. Subsequently, the cells were washed with cold PBS to remove unincorporated radioactivity. Radiolabeled neutrophils (1 × 10⁶/well) were added to HUVEC monolayers, and 30 minutes later the percentage of added PMNs that remained adherent after a wash procedure was quantitated using a standard approach.

Electrophoretic Mobility Shift Assay

HUVECs grown in 48-well plates were scraped for preparation of nuclear extracts for electrophoretic mobility shift assay (EMSA) as previously described. The double-stranded oligonucleotide containing consensus (5′-AGGAACTTTCCGCTGGGACTTTC-3′) binding sites for NF-κB (provided by Dr T. Archer) was labeled with [γ-³²P]ATP (Amersham Canada Ltd) by using T4 polynucleotide kinase (MBI Fermentas Inc) as previously described. One picomole of the labeled oligonucleotide was incubated with 5 μg of nuclear protein in the presence or absence of a 50-fold excess of cold oligonucleotide for 30 minutes, and the reaction mixture was then loaded onto native 5% polyacrylamide gel and electrophoresed at 250 V in 0.5 × Tris-borate EDTA buffer. The dried gels were then exposed to x-ray films (Kodak) for 16 hours in cassettes with intensifying screens.

Nitrite/Nitrate (NO₇⁻/NO₃⁻)

HUVEC production of NO was determined indirectly by measuring NO₇⁻/NO₃⁻ concentration in HUVEC supernatants as previously described. Briefly, supernatants (100 μL) obtained from HUVECs after treatments (using M199 medium without phenol red) were collected and incubated for 30 minutes at 37°C in the presence of 0.2 U/mL of Aspergillus nitrate reductase (Boehringer Mannheim Canada), 50 mmol/L HEPES buffer, (pH 7.4), 5 μmol/L flavin adenine dinucleotide, and 0.1 mmol/L NADPH. Subsequently, lactate dehydrogenase (Boehringer Mannheim Canada) and sodium pyruvate (Sigma Chemical Co) were added to a final concentration of 10 U/mL and 10 mmol/L, respectively, and the samples were incubated for an additional 10 minutes at 37°C. The Griess reagent was added to the samples (100 μL), and after an additional 15-minute incubation at room temperature, absorbance was read at 540 nm. As a standard, sodium nitrite solution (1 to 50 μmol/L) in M199 was used.

Western Blot of NOS

After treatments, HUVEC monolayers were lysed using a hot 2× concentrated electrophoresis sample buffer (1× = 125 mmol/L Tris-HCl, pH 6.8, 2% SDS, and 5% glycerol). Protein concentration of the samples was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added to the samples at a final concentration of 1% vol/vol, and they were denatured by boiling for 5 minutes. Ten micrograms of protein was electrophoresed on 7% SDS-polyacrylamide gel and transferred to reinforced nitrocellulose membranes (Schleicher & Schuell, Inc) using an electrophoretic transfer unit ( Hoefer, Inc) at a constant current of 1 A for 3 hours in a transfer buffer containing 25 mmol/L Tris,
190 mmol/L glycine, and 20% methanol. Blots were incubated overnight in a blocking solution (3% BSA and 0.05% Tween 20 in PBS). Subsequently, the membranes were treated with mouse anti-human endothelial NOS (eNOS) monoclonal antibody (mAb) or a mouse anti-macrophage NOS antibody (inducible NOS [iNOS] mAb) (Transduction Laboratories Inc). The NOS mAb binding was detected using biotinylated anti-mouse IgG and Vectastain Elite avidin-biotin complex–peroxidase detection system (Vector Laboratories Inc).

**Reactive Oxygen Intermediates**

To assess oxidant production within endothelial cells, we used a quantitative measure of H2O2-derived oxidant formation by monitoring the oxidation of dihydrorhodamine 123 (DHR 123) (Molecular Probes, Inc), an oxidant-sensitive fluorochrome.15 HUVEC monolayers were treated with DHR 123 (5 μmol/L) in phenol red-free M199 for 1 hour before experiments. After treatments, supernatants were removed, and cells were washed with PBS and lysed in a buffer containing 0.1% CHAPS, 50 mmol/L K2HPO4 (pH 7.0), and 0.1 mmol/L EDTA. The cell lysates were sonicated at a 30% power output for 1 minute and centrifuged at 2000 g for 10 minutes at 4°C. The clarified cell sonicates were diluted 40 times and analyzed for DHR 123 oxidation (rhodamine fluorescence) at excitation and emission wavelengths of 502 and 523 nm, respectively. The fluorescence intensity was expressed as optical units per μg of cell protein.

**Inhibitors**

A double-stranded (ds) phosphorothioate oligonucleotide (Bio-Synthesis, Inc) containing a consensus NF-κB binding site (sequence of the sense strand is 5'-AGGGACTTTCCGCTGGGGACTTTCC-3', and the antisense strand represents the reverse complement) was used to inhibit NF-κB translocation to HUVEC nuclei.16 As a control, mutant ds-phosphorothioate oligonucleotide (sense strand 5'-ACTCACTTTCCGCTGCTCACTTTCC-3') was used under identical conditions (mutated sites are underlined). In addition, a proteasome inhibitor, MG 132 (MyoGenics, Inc) was used to block NF-κB activation.17 Standard NOS inhibitors were used to assess the role of NO. Nω-nitro-l-arginine methyl ester (L-NAME) or its inactive entionomer, D-NAME (Sigma Chemical Co) was applied to HUVECs under various experimental conditions using l-arginine-free M199 medium. l-Arginine was used to counteract the effects of L-NAME. An inhibitor of GTP-cyclohydrolase I, 2,4-diamino-6-hydroxypyrimidine (DAHP) (Sigma Chemical Co), was used in some experiments.

**Statistical Analysis**

All values are presented as mean±SEM. Each experiment was performed in triplicate. Statistical analysis was performed using ANOVA and Student’s t test (with Bonferroni corrections for multiple comparisons). P<0.05 was considered statistically significant.

**Results**

**In Vitro Model of I/R Tolerance**

We have adapted our in vitro model of A/R-induced inflammation1 to assess some of the cellular mechanisms involved in the development of A/R tolerance in endothelial cells. Exposing HUVECs to anoxia for 30 minutes and adding neutrophils (PMNs) upon reoxygenation resulted in an increase in PMN adhesion to HUVECs (Figure 1A). This observation is consistent with our previous studies2 and closely mimics the situation in vivo, in which leukocytes adhere to venular endothelium after reperfusion of ischemic tissues.1,2

To mimic the in vivo models of I/R tolerance, HUVECs were exposed to a 30-minute period of anoxia and then reoxygenated for different periods of time. Subsequently, HUVECs were exposed to a second A/R challenge, at which time PMN adhesion to HUVECs was assessed. As shown in Figure 1A, when HUVECs were exposed to an A/R insult and...
then subsequently challenged with a second A/R insult (24 hours later), the level of PMN adhesion to HUVECs after the second A/R insult was similar to that observed in normoxic controls. This decrease in PMN adhesion was not due to an adverse effect on endothelial cell viability (trypan blue exclusion). These findings indicate that HUVECs mounted an adaptational response to the first A/R insult, which prevented the hyperadhesiveness of PMNs to HUVECs after the second challenge. We have termed this adaptational response “A/R tolerance.”

Further characterization experiments indicated that the A/R tolerance (with respect to PMN adhesion to HUVECs) did not occur if the hiatus between the 2 A/R challenges was reduced to 1 or 6 hours (Figure 1B). These latter observations suggested that a transcriptional event may be involved in the development of A/R tolerance in our model. We focused our attention on the nuclear transcription factor NF-κB, which has been implicated in the regulation of number of genes whose products modulate inflammation.18,19

**Role of NF-κB in the Development of A/R Tolerance**

To assess whether A/R activates NF-κB and, thereby, promotes its translocation to the nuclei of HUVECs, we used an EMSA to detect NF-κB in nuclear extracts of HUVECs obtained at different times after the initial A/R insult. As shown in Figure 2A, there was no significant increase in HUVEC nuclear NF-κB at 30 minutes after reoxygenation. Since PMN adhesion increased within 30 minutes after reoxygenation, these findings indicate that it is unlikely that NF-κB plays a role in the hyperadhesive response to the initial A/R challenge in our model. However, NF-κB was present in the nuclei of HUVECs by 4 hours after the initial A/R insult and, therefore, may be involved in the development of A/R tolerance.

To assess whether activation of NF-κB plays a role in the development of A/R tolerance, we used 2 experimental approaches. First, we pretreated HUVECs with a proteasome inhibitor (MG 132) that has previously been shown to inhibit NF-κB activation.17 MG 132 was added to HUVECs 30 minutes before the initial A/R insult and removed 30 minutes after reoxygenation. As shown in Figure 2B, this maneuver prevented the development of A/R tolerance; ie, PMN adhesion to HUVECs after the second A/R insult was similar to that observed after an A/R insult imposed on nontolerant cells (N/R, A/R in Figure 2B). Pretreatment of HUVECs with MG 132 alone (no initial A/R challenge) did not affect the increase in PMN adhesion induced by an A/R challenge imposed 24 hours later (data not shown). Another approach was to pretreat HUVECs with a phosphorothioate oligonucleotide, (pt)NF-κB, containing a consensus binding sequence for NF-κB for 3 hours before the initial A/R challenge. This maneuver has previously been shown to inhibit NF-κB translocation to HUVEC nuclei.16 As shown in Figure 2B, (pt)NF-κB was just as effective as the proteasome inhibitor in preventing the development of A/R tolerance. Pretreatment with a mutated (pt)NF-κB oligonucleotide was ineffective in preventing the development of A/R tolerance. These 2 approaches have previously been shown to be effective in preventing the translocation of NF-κB to nuclei of HUVECs exposed to A/R.4 In addition, in the present study these 2 inhibitors prevented NF-κB appearance in HUVEC nuclei 4 hours after the initial A/R challenge (data not shown).

**Role of NOS in the Development of A/R Tolerance**

One anti-inflammatory gene that is activated by NF-κB is the one encoding iNOS. NOS is responsible for the production of NO, a potent endogenous inhibitor of PMN adhesion to endothelium.20 To assess whether NO may play a role in the development of A/R tolerance, we measured the concentrations of NO2⁻/NO3⁻ (Griess reaction) in supernatants obtained from HUVECs 30 minutes after an A/R challenge. As shown in Figure 3A, the NO2⁻/NO3⁻ concentration in supernatants of HUVECs exposed to an initial A/R challenge were not different from those observed in normoxic controls. However, NO2⁻/NO3⁻ levels were increased in response to an A/R

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**Table 2.** Role of NF-κB in the development of A/R tolerance with respect to PMN adhesion to HUVECs. A. Effect of A/R on nuclear appearance of NF-κB in HUVECs. HUVECs were exposed to A/R, and 0.5, 4, and 12 hours after reoxygenation, nuclear extracts were obtained for EMSA. Five micrograms of extracted protein were incubated with 1 pmol of γ-32P-labeled oligonucleotide containing consensus NF-κB–binding sites and subjected to 5% PAGE under nondenaturing conditions (see Materials and Methods for details). A representative experiment is shown; 2 additional experiments yielded similar results. NF-κB in nuclear extracts of HUVECs became apparent by 4 hours after reoxygenation. B. Effects of NF-κB inhibitors on PMN adherence to A/R-tolerant HUVECs. The experimental conditions are the same as those described in Figure 1. In some experiments, HUVECs were pretreated for 3 hours with 20 μmol/L ds-phosphorothioate oligonucleotide containing binding sites for NF-κB, (pt)NF-κB, or a mutated oligonucleotide, ∆(pt)NF-κB, before the initial A/R. In other experiments, HUVECs were treated with 30 μmol/L MG 132, a proteasome inhibitor, during the initial A/R and removed 30 minutes after reoxygenation. Both (pt)NF-κB and MG 132 prevented the development of A/R tolerance with respect to PMN adhesion. n=5. 10 P<0.05 as compared with N/R, A/R.
challenge imposed on tolerant HUVECs (exposed to an initial A/R challenge 24 hours previously).

Since NO production was increased in tolerant HUVECs, we assessed the role of NO in the development of A/R tolerance. L-NAME, an NOS inhibitor, was added to HUVECs before the A/R challenges. As shown in Figure 3B, L-NAME had no effect on basal PMN adhesion to HUVECs (normoxic controls) or the hyperadhesion induced after the initial A/R insult. However, L-NAME prevented the A/R tolerance with respect to PMN adhesion (ie, the decreased adhesion response usually noted after the second A/R challenge was no longer observed). The coadministration of L-arginine prevented this effect of L-NAME. D-NAME had no effect on the A/R-induced PMN adhesion to HUVECs in tolerant cells. Taken together, these findings indicate that the generation of NO by HUVEC NOS plays an important role in the development of tolerance with respect to PMN adhesion.

To determine whether extracellularly generated NO could be as effective as intracellularly generated NO in preventing PMN adhesion to HUVECs, we used a NO donor. Spermine NONOate (Molecular Probes; 0.1 mmol/L final concentration) was added along with the PMNs after the initial A/R challenge, and PMN adhesion was assessed. Although spermine NONOate increased the concentration of NO₂⁻/NO₃⁻ in the supernatant (Griess reaction) to levels (16 μmol/L) greater than those observed after the second A/R challenge (Figure 3), the NO donor did not prevent the A/R-induced increase in PMN adhesion to HUVECs (Figure 4).

Western blot analyses of HUVECs under basal conditions (normoxic controls) or after A/R challenges indicated that HUVECs lack iNOS (data not shown). This observation confirms previous reports that HUVECs do not contain iNOS.²¹ By contrast, constitutive eNOS was present both under basal conditions and after the A/R challenges (Figure 5). Interestingly, eNOS protein was increased after an A/R challenge to both nontolerant and tolerant HUVECs. From the data presented in Figure 5, it appears that the increase in eNOS protein induced by the initial A/R insult had returned to near basal levels within 24 hours and that the second A/R challenge induced a comparable increase in eNOS protein as observed after the initial A/R insult.

Role of Tetrahydrobiopterin (BH₄) in the Development of A/R Tolerance

Previous studies have indicated that BH₄ is an important co-factor for eNOS activity in HUVECs,²¹,²² BH₄ is synthesized by 2 pathways, which are de novo synthesis from GTP and a pterin salvage pathway that regenerates BH₄ from

Figure 3. Role of NO in the development of A/R tolerance. A, Generation of NO in A/R-tolerant HUVECs. NO production was assessed by measuring the concentration of NO₂⁻/NO₃⁻ (Griess reaction) in the supernatants obtained from HUVECs after exposure to normoxic conditions (N/R, N/R), a first A/R challenge (N/R, A/R), and 2 A/R challenges separated by a 24-hour hiatus (A/R, A/R). An increase in NO production above normoxic controls was found in A/R-tolerant HUVECs (A/R, A/R). n=5. *P<0.05 as compared with N/R, A/R. B, Effects of an NOS inhibitor (L-NAME) on PMN adhesion to A/R-tolerant HUVECs. The experimental conditions are the same as those described in Figure 1. HUVECs were exposed to normoxic conditions (N/R, N/R), a first A/R challenge (N/R, A/R), and a second A/R challenge administered 24 hours after the first A/R challenge (A/R, A/R). HUVECs were treated with L-NAME (100 μmol/L) during the second challenge. The specificity of L-NAME was assessed by introducing L-arginine (300 μmol/L) to the cells along with L-NAME or treating HUVECs with D-NAME (100 μmol/L). n=6. L-NAME prevented the development of A/R tolerance with respect to PMN adhesion. *P<0.05 as compared with N/R, A/R.
dihydrobiopterin. 

The rate-limiting step in the de novo synthesis pathway involves the enzyme GTP-cyclohydrolase I. Thus, we assessed the effects of DAHP, an inhibitor of GTP-cyclohydrolase I, on NO production by HUVECs. As shown in Figure 6, pretreatment of HUVECs with DAHP (5 mmol/L) dramatically reduced NO production by HUVECs under basal conditions (normoxic controls) and after an A/R insult imposed on naive or tolerant HUVECs. The reduced NO production was not due to a DAHP-induced adverse effect on HUVEC viability (trypan blue exclusion). These findings indicate that NO production under basal conditions and after the initial A/R insult are dependent on BH4 synthesis by GTP-cyclohydrolase I. More importantly, the increase in NO production usually observed after the second A/R challenge was completely abolished.

Interaction of NO With Oxidants

Supernatants from A/R-conditioned HUVECs can increase PMN adhesion to naive HUVECs, an effect prevented by coadministration of catalase in the adhesion assay. Furthermore, exogenous administration of oxidants to endothelial cells can promote PMN adhesion. Taken together these observations indicated that exposing HUVECs to A/R induces an oxidant stress within HUVECs, which may promote PMN adhesion to HUVECs. Thus, a possible explanation for why HUVECs become tolerant to a second A/R challenge may be that HUVECs are subjected to less oxidant stress during the second challenge. To test this hypothesis, HUVECs were preloaded with DHR 123 for 1 hour before challenge with A/R. As shown in Figure 7A, the first A/R challenge was associated with an increase in DHR 123 oxidation, indicating that an intracellular oxidant stress occurred in HUVECs. By contrast, DHR 123 oxidation was not increased by the second A/R challenge imposed 24 hours after the initial A/R insult. DHR 123 uptake by HUVECs was the same when loaded before the first or second A/R challenge. These findings indicate that the development of A/R tolerance is associated with a decrease in the ability of A/R to generate an oxidant stress in HUVECs.

Previous studies indicate that NO can interact with oxidants. Thus, we next tested the possibility that NO generated during the second A/R challenge was involved in reducing the oxidant stress incurred during the second A/R challenge. As shown in Figure 7B, administration of L-NAME during the initial A/R insult had no effect on DHR 123 oxidation. However, the presence of L-NAME during the second A/R challenge prevented the decrease in DHR 123 oxidation typically seen after the second challenge (compare with Figure 7A).

To assess whether the increased NO production and the suppression of oxidative stress observed in HUVECs after the second A/R challenge was mediated by NF-κB, we used the proteasome inhibitor MG 132. The proteasome inhibitor was added to HUVECs 30 minutes before the initial A/R insult and removed 30 minutes after reoxygenation. After the second A/R challenge either NO2⁻/NO3⁻ levels in the supernatant were measured (Griess reaction) or oxidant stress in

![Figure 5. Role of NOS in the development of A/R tolerance. A, Analysis of eNOS protein in HUVECs (Western blot). Cell lysates were obtained from HUVECs exposed to normoxia (N/R, N/R), an A/R challenge (N/R, A/R), or 2 A/R challenges (A/R, A/R) or from HUVECs challenged with an initial A/R insult and reoxygenated for 24 hours (A/R, N/R). Ten micrograms of total protein were subjected to 7% SDS-PAGE and transferred to nitrocellulose membranes. The blots were treated with mouse anti-human eNOS mAb, a biotinylated secondary antibody, and evaluated using an avidin-biotin complex–peroxidase detection system. Shown is a representative assay of 8 separate experiments. B, Densitometric analysis of eNOS Western blots. The experimental conditions are the same as those described in panel A. Values are averages of 8 independent experiments. The results indicate that A/R increases eNOS protein in both A/R-tolerant and -nontolerant HUVECs. *P<0.05 as compared with N/R, N/R. #P<0.05 as compared with A/R, N/R.](http://circres.ahajournals.org/issue/1/9/108)

![Figure 6. The effects of DAHP on the production of NO in A/R-tolerant HUVECs. The experimental conditions are the same as those in Figure 3A. HUVECs were challenged with normoxia (N/R, N/R), A/R (N/R, A/R), or 2 A/R episodes (A/R, A/R). In some experiments 5 mmol/L DAHP, a GTP-cyclohydrolase I inhibitor, was applied to HUVECs 30 minutes before the first challenge. n=4. DAHP decreased NO production under all conditions. *P<0.05 as compared with respective treatments without DAHP.](http://circres.ahajournals.org/issue/1/9/108)
HUVECs was assessed (DHR 123 oxidation). As shown in Figure 8A, pretreatment with MG 132 ameliorated the increase in NO\(_2^–/NO_3^–\) levels typically seen after the second A/R challenge. In addition, the suppression in oxidative stress typically noted after the second A/R challenge did not occur (Figure 8B).

**Discussion**

Exposure of HUVECs to A/R (30 minutes of anoxia and 30 minutes of reoxygenation) results in an increase in PMN adhesion to HUVECs.\(^3\) This endothelial cell response to A/R is not due to an increase in HUVEC adhesivity (ie, surface levels of adhesion molecules, such as the cytokine-regulated intercellular adhesion molecule-1 and E-selectin are not increased).\(^3\) Rather, this hyperadhesive response is due to soluble factors produced and released by the endothelial cells (H\(_2\)O\(_2\) and PAF), which subsequently activate PMNs and
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enhance their ability to adhere to HUVECs. In the present study, we provide evidence that HUVECs can develop A/R tolerance with respect to A/R-induced PMN adhesion to HUVECs. Our findings indicate that the A/R-induced increase in PMN adhesion to HUVECs can be completely abolished by pretreating HUVECs with an A/R challenge 24 hours previously (Figure 1). This adaptational response requires more than 6 hours to develop (Figure 1B), indicating that some transcriptional event may be involved.

We focused our attention on the nuclear transcription factor NF-κB, since it regulates the activity of a variety of genes whose products modulate inflammation. For example, activation of NF-κB leads to the transactivation of proinflammatory genes, such as those encoding (1) endothelial adhesion molecules involved in PMN adhesion to endothelial cells and (2) cytokines and growth factors. However, more relevant to the development of A/R tolerance in our model are the anti-inflammatory genes activated by NF-κB. The potential anti-inflammatory effects of NF-κB activation, coupled to the ability of PAF and H₂O₂ (factors implicated in the increased PMN adhesion to HUVECs in our model of A/R) to activate NF-κB, prompted us to assess the potential role of NF-κB in the development of A/R tolerance in our system.

In endothelial cells, NF-κB exists as a heterodimer consisting of subunits designated as p50 and p65. It is localized to the cytoplasm in an inactive form by virtue of its association with a monomeric inhibitory protein, IκB. Activation of NF-κB occurs by phosphorylation, ubiquitination, and subsequent degradation of IκB by the proteasome, a multicatalytic protease. The loss of IκB allows the NF-κB heterodimer to translocate to the nucleus and initiate the transcription of genes regulating the inflammatory process. As shown in Figure 2A, NF-κB is activated by the initial A/R insult, since it appears within HUVEC nuclei within 4 hours. Furthermore, a proteasome inhibitor (MG 132), which has been shown to inhibit activation of NF-κB in HUVECs, prevented the development of A/R tolerance with respect to PMN adhesion to HUVECs (Figure 2B). Finally, interfering with NF-κB translocation to the nuclei by using a double-stranded oligonucleotide containing NF-κB binding sites also prevented the development of A/R tolerance (Figure 2B).

Taken together, our findings indicate that activation and translocation of NF-κB to the nuclei of HUVECs is an important component of the development of A/R tolerance. Interestingly, NF-κB was detected in HUVEC nuclei at 4 hours after the initial A/R challenge (Figure 2A), whereas the development of A/R tolerance with respect to PMN adhesion was not detected as late as 6 hours after the initial A/R challenge (Figure 1B). The reason for the delay in the development of A/R tolerance is not clear, but delay may be due to the time required for NF-κB to induce transactivation of relevant gene(s) and subsequent protein synthesis.

The gene(s) that NF-κB transactivates within HUVECs in order for the anti-inflammatory phenotype to be manifested in A/R-tolerant cells (decreased PMN adhesion) is unclear. Since NO is an endogenous antiadhesive molecule, genes regulating NO production are possible candidates. Thus, we first examined the possibility that an increased production of NO was involved in the development of A/R tolerance. Our findings indicate that, while the first A/R insult is not associated with increased NO production, the second A/R challenge is (Figure 3A). Furthermore, inhibition of NOS activity with L-NAME during the second A/R challenge prevented the development of A/R tolerance with respect to PMN adhesion to HUVECs (Figure 3B). These findings indicate that an increase in NO production during the second A/R challenge contributes to the manifestation of A/R tolerance.

One likely gene target for NF-κB that would enhance NO production during the second A/R challenge is iNOS, which contains NF-κB–binding sites in its promoter region and is transactivated by NF-κB. However, Western blot analyses indicated that iNOS was not present in HUVECs under basal conditions or after challenge with A/R. These observations are consistent with a previous report indicating that HUVECs do not contain iNOS. On the other hand, eNOS was present under basal conditions and was increased by both the initial and second A/R challenges (Figure 5). The mechanisms involved in the A/R-induced increase in eNOS protein observed in the present study are unclear. Previous reports have noted that eNOS message and protein can be increased by mechanical, shear, and oxidative stresses applied to endothelial cells. The modulation of eNOS message and protein levels appear to involve transcriptional and/or posttranscriptional events. Thus, it could be argued that the development of A/R tolerance involves NF-κB–induced transcription of eNOS. That this is most likely not the case is based on the following lines of evidence. First, although SP-1 and activator protein-1–binding elements have been identified in the promoter region of the human eNOS gene, there does not appear to be an NF-κB–binding site on the gene encoding eNOS. Second, while eNOS protein is increased within 30 minutes of reoxygenation (Figure 5), there is no detectable NF-κB in the nucleus at this time (Figure 2). Third, although eNOS protein was increased during the initial A/R (Figure 5), there was no corresponding increase in NO production (Figure 3A). Thus, the NF-κB–mediated development of A/R tolerance (with respect to NO production and PMN adhesion) most likely involves some other transcriptional event besides induction of eNOS.

BH₄ is an important cofactor for NOS activity. In the present study, inhibition of GTP-cyclodihydrolase I, the enzyme responsible for de novo synthesis of BH₄, completely abolished NO synthesis by HUVECs (Figure 6). Of particular relevance to the present study is the observation that the gene encoding for GTP-cyclodihydrolase I contains an NF-κB–binding site in the promoter region. In addition, cytokine-induced NO production by endothelial cells, myocytes, and smooth muscle cells is dependent on GTP-cyclodihydrolase I activity. Thus, taken together, our findings suggest that NF-κB may contribute to the development of A/R tolerance by transactivating the gene encoding GTP-cyclodihydrolase I. Further studies are warranted to more directly address this possibility.

One interesting observation in the present study was that there appears to be an interaction between NO and oxidants in HUVECs during the development of A/R tolerance. The
initial A/R insult induced an oxidant stress, while the subsequent A/R challenge did not (Figure 7A). Furthermore, with addition of the NOS inhibitor (L-NAME) during the second A/R challenge, the degree of A/R-induced oxidant stress was similar to that observed after the initial A/R challenge. These findings indicate that the increased NO production during the second A/R challenge reduces the oxidant stress typically induced by A/R. This contention is consistent with other reports showing that, when NO production exceeds oxidant production, oxidant-mediated lipid peroxidation or hydroxylation of benzoate is substantially inhibited. It is not entirely clear whether the decreased PMN adhesion to A/R-tolerant HUVECs is due directly to an increased NO production or the fact that NO decreases the oxidant stress. However, since exogenous generation of NO (NO donor; spermine NONOate) during the initial A/R challenge did not prevent the hyperadhesion response (Figure 4), it is unlikely that NO directly inhibits PMN adhesion to HUVECs. Thus, the most likely explanation for our findings is that intracellularly generated NO inhibits PMN adhesion during the second A/R challenge by reducing the oxidant stress.

Figure 9 schematically depicts our working hypothesis on the mechanisms involved in the development of A/R tolerance in HUVECs. We propose that after the initial A/R insult an oxidant stress is generated within HUVECs that leads to an increase in PMN adhesion to HUVECs (Figures 1 and 7). On the basis of our previous studies and those of others, it is likely that H2O2 plays an important role in this hyperadhesion via generation of PAF. In addition, the initial A/R insult induces a transcriptional event that leads to the development of A/R tolerance with respect to A/R-induced PMN adhesion to HUVECs. Specifically, NF-κB is activated and translocates to the nucleus (Figure 2). NF-κB then promotes the transcription of a relevant gene(s), which results in an increase in NO production by eNOS during the second A/R challenge. Our preliminary findings (inhibitor studies) indicate that NF-κB may transactivate the gene encoding GTP-cyclohydrolase I, which subsequently results in the synthesis of BH4, an important cofactor for eNOS activity (Figure 6). The increase in NO production during the second A/R challenge prevents the oxidant stress within HUVECs (Figures 7 and 8). The decrease in PMN adhesion to HUVECs after the second A/R challenge may be a result of the lack of an oxidant stress and associated PAF production. In the present study we also provide evidence that NF-κB is involved in the increased NO production and the suppression of the A/R-induced oxidant stress (Figure 8). Although the results of the present study support various aspects of this working hypothesis, further studies are necessary to firmly establish the relative roles of various proposed factors in the development of A/R tolerance.

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