Oxidative Stress Induces NF-κB Nuclear Translocation Without Degradation of IκBα

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Background—Rel/NF-κB, an oxidative stress–responsive transcription factor, participates transiently in the control of gene expression. The cellular mechanisms that mediate NF-κB activation during ischemia (and during reperfusion in the course of treating ischemia) are not known.

Methods and Results—To investigate the NF-κB activation induced during oxidative stress, we examined human umbilical vein endothelial cells obtained during surgical procedures requiring cardiopulmonary bypass. In vitro, we examined human umbilical vein endothelial cells (HUVECs) exposed to hypoxia, reoxygenation after hypoxia, or a reactive oxygen intermediate (H2O2). Western blotting of cytoplasmic IκBα demonstrated that NF-κB activation induced by oxidative stress was not associated with IκBα degradation. In contrast, tumor necrosis factor-α–induced NF-κB activation occurred in concert with degradation of IκBα. Western blotting of cytoplasmic IκBα demonstrated that NF-κB activation induced by oxidative stress was not associated with IκBα degradation. In contrast, tumor necrosis factor-α–induced NF-κB activation occurred in concert with degradation of IκBα.

Conclusions—This study demonstrated a stimulus-specific mechanism of NF-κB activation in endothelial cells that acts independently of IκBα degradation and may require tyrosine phosphorylation. (Circulation. 1999;100[suppl II]:II-361–II-364.)

Key Words: cardiopulmonary bypass ■ ischemia ■ reperfusion ■ endothelium

Reperfusion of ischemic tissue creates a pathophysiological paradox: although reperfusion is necessary to prevent further ischemic necrosis, it may damage or cause dysfunction in otherwise viable tissue; this is referred to as ischemia-reperfusion (I/R) injury.1 I/R injury contributes significantly to morbidity and mortality in patients with ischemic heart disease, stroke, peripheral vascular insufficiency, early transplant organ dysfunction, and hemorrhagic shock. On a cellular level, the reperfusion of ischemic tissue creates oxidative stress.

During reperfusion, endothelial cells are critical in recruiting and activating neutrophils, the principal cause of acute inflammation.2 In response to septic insults, endothelial cells undergo a phenotypic change leading to the synthesis of neutrophil adhesion molecules (eg, E-selectin [CD62E]) and neutrophil chemotactic agents (eg, interleukin [IL]-8).3,4 This transient transcriptional response is mediated, in part, by Rel/nuclear factor κB (NF-κB).5 In quiescent endothelium, NF-κB is held inactive within the cytoplasm by interaction with inhibitor κB (IκBα) or other IκB family members.6 After stimulation with tumor necrosis factor (TNF)-α, lipopolysaccharide (LPS), or IL-1, IκBα undergoes rapid phosphorylation on serine residues 32 and 36; this signals the proteolytic degradation of IκBα in proteasomes.7 The dissociation of IκBα from NF-κB exposes the nuclear localization signal8 on NF-κB, which directs the translocation of the transcription factor to the nucleus to promote the expression of genes that mediate inflammatory reactions.9 NF-κB–mediated regulation of inflammatory gene expression in human endothelial cells during oxidative stress, however, is not defined.

In this study, we demonstrated that NF-κB activation occurs in human hearts subjected to ischemia and reperfusion during surgical procedures using cardiopulmonary bypass. In addition, we showed in vitro that NF-κB activation occurs in cultured human endothelial cells treated with a reactive oxygen intermediate (H2O2) and in cultures exposed to hypoxia followed by reoxygenation. Unlike NF-κB activation induced by TNF-α, IL-1, or LPS, NF-κB activation under conditions of oxidative stress is achieved without measurable degradation of IκBα and may involve tyrosine phosphorylation.
Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were obtained by collagenase (Whorton Biochemical) digestion, as previously described. Cultures were maintained in RPMI 1640 solution (Whittaker) supplemented with 20% adult bovine serum (JRH Biosciences), 90 U/mL heparin (Sigma), 50 U/mL endothelial cell growth supplement (Collaborative Research), and 1% antibiotics in room air at 37°C. HUVECs were serially passaged by brief exposure to Versene (GIBCO/BRL) and 0.05% trypsin with EDTA (GIBCO/BRL). Third-passage confluent HUVEC monolayers were used in all experiments.

Human Cardiac Tissue Procurement
Right auricular tissue from patients undergoing coronary artery bypass grafting was obtained during cannulation of the right atrium in preparation for cardiopulmonary bypass (CPB). Skeletal muscle samples were obtained from the intercostal muscle. Samples were obtained before the start of cardiopulmonary bypass and 45 minutes after the patient was taken off bypass. All tissues were immediately snap-frozen in liquid nitrogen in the operating room. Frozen tissue samples, 0.25 to 0.5 g, were then ground to a fine powder and suspended in 4.0 mL of buffer containing 0.06% Nonidet P-40, 0.15 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA, and 0.5 mmol/L PMSF. The solution was then homogenized and centrifuged for 15 s. The pellet was discarded, and the supernatant was cooled to 4°C. The supernatant was then centrifuged again for 15 s. The pellet was resuspended in 40 µL of buffer containing (in mmol/L): NaCl 420, HEPES 20, EDTA 0.2, MgCl2 1.2, PMSF 0.5, and DDT 0.5 and 25% glycerol, 5 µg/mL aprotinin, and 5 µg/mL leupeptin at 4°C for 20 minutes. This solution was then centrifuged for 5 minutes, the pellet was discarded, and the supernatant, containing nuclear proteins, was frozen at −70°C. Samples were analyzed for NF-κB as described below. This study was approved by the Human Subjects Committee of the University of Washington.

Hypoxia and Reoxygenation
Normoxic control cells were maintained at 37°C in a standard incubator at room air oxygen tension (21% oxygen; designated normoxia). To expose HUVECs to hypoxia, cultures were placed in a controlled environmental chamber (Coy Laboratory Products) at 37°C and maintained at an oxygen tension of 2% to 3%. Oxygen content in the media over HUVECs equilibrated with the hypoxic environment of the chamber within 10 minutes, as measured by a Clark electrode (data not shown). To expose HUVECs to hypoxia and reoxygenation, cultures were removed from the hypoxia chamber and placed in a normoxic incubator for designated periods of time.

Electrophoretic Mobility Shift Assay
Third-passage HUVEC monolayers in 100-mm² plates were exposed to 2 hours of hypoxia followed by reoxygenation, H2O2 (100 to 500 µmol/L), pervanadate (100 to 200 µmol/L), or TNF-α (100 U/mL) for designated time periods. Nuclear protein extractions were performed using a modification of the technique of Dignam et al., as previously described. Approximately 10 µg of nuclear protein was incubated in a binding reaction with a double-stranded, 32P-end-labeled oligonucleotide containing the human consensus NF-κB binding sequence 5′-GCCATTTGAGATCTTCTTTATCGG-3′ (Promega). Binding reactions occurred at room temperature for 20 minutes. Proteins were resolved on 6% nondenaturing polyacrylamide gels at 100 V for 1 to 2 hours in a 0.5% Tris-boric acid-EDTA solution. The gels were dried and autoradiographed.

Western Blot Analysis
Confluent HUVEC monolayers on 100-mm² plates were treated with conditions of oxidative stress or TNF-α in the manner described above. Cytoplasmic extracts were obtained using a modified lysis buffer, and total protein concentration was determined using a standard protein assay. Approximately 20 µg of protein was loaded on SDS-PAGE gels and resolved at 100 V for 2 hours. After transfer to a polyvinylidene difluoride membrane, the membrane was stained with Coomassie blue to determine equal protein transfer. The membranes were then incubated with anti-1k-β polyclonal antibody (Santa Cruz Biotechnology) at 1:1000 dilution for 2 hours. A HRP-conjugated secondary antibody was applied for 1 hour, and the proteins were visualized using Amersham enhanced chemiluminescent reagents and autoradiography.

Results
To determine the activation of NF-κB in human tissue under conditions of oxidative stress, cardiac and skeletal muscle samples were acquired before patients were placed on CPB and after reperfusion of the heart for 45 minutes, after the patient was taken off CPB. We chose right atrial tissue because this tissue receives the least amount of cardioplegia during CPB, and the anterior location of the right atrium makes it less likely to be cooled to a temperature approaching 4°C; previously, we demonstrated that NF-κB can be activated at temperatures lower than 37°C but not at those ≤4°C. As shown in Figure 1, NF-κB was prominently activated in human atrial tissue after 45 minutes of reperfusion of the ischemic heart. However, skeletal muscle obtained from the same patient at the same time points but not exposed to ischemia and reperfusion showed no evidence of NF-κB activation.

To define more specifically mechanisms of oxidative stress–induced NF-κB activation, HUVECs were reoxygenated after a 2-hour period of hypoxia and treated with H2O2 or pervanadate. Also, HUVECs were treated with medium alone or medium containing TNF-α (100 U/mL) for 2 hours as a control stimulus of NF-κB activation. In each experiment, either nuclear proteins were extracted for electrophoretic mobility shift assays (EMSAs) to determine NF-κB activation or cytoplasmic proteins were extracted for Western blotting to determine the presence or absence of 1k-β. As shown in Figure 2A, TNF-α induced rapid degradation of 1k-β protein, with concomitant nuclear translocation of NF-κB within 5 to 10 minutes of the addition of TNF-α;
complete IκBα degradation was noted by 15 minutes, with the reappearance of IκBα in the cytoplasm by 1 hour during continuous exposure to TNF-α. Similar results were obtained when cells were treated with IL-1 (10 U/mL) or LPS (100 ng/mL) (data not shown). HUVECs exposed to 2 hours of hypoxia followed by reoxygenation for various periods of time also demonstrated rapid nuclear translocation of NF-κB within 15 minutes after reoxygenation was started (Figure 2B). Similarly, rapid nuclear translocation of NF-κB occurred in HUVECs treated with H2O2 (Figure 2C). However, in contrast to the treatment of HUVECs with TNF-α, reoxygenation of hypoxic HUVECs (Figure 2B) and treatment of HUVECs with H2O2 (Figure 2C) did not result in degradation of IκBα.

Tyrosine phosphorylation of IκBα inJurkat T-cells treated with pervanadate reportedly prevents IκBα degradation.13 Pervanadate, a tyrosine phosphatase inhibitor, is composed of H2O2 and vanadate. Pervanadate thus preserves transient tyrosine phosphorylation of proteins and potentiates signal transduction pathways involving tyrosine kinases. As shown in Figure 2D, pervanadate treatment initiated rapid and pronounced nuclear translocation of NF-κB, without associated degradation of IκBα. To exclude the possibility that oxidative stress induces NF-κB activation through the degradation of NF-κB cytoplasmic inhibitors other than IκBα, Western blots (using polyclonal antiser to IκB family members IκBβ or IκBε) were performed on cytoplasmic proteins from HUVECs treated with pervanadate. Neither IκBβ nor IκBε were degraded in HUVECs exposed to oxidative stress (data not shown).

Because oxidative stress seems to activate NF-κB without apparent degradation of IκB proteins, we reasoned that inhibitors of IκB degradation in the proteasome should have no effect on oxidative stress-induced NF-κB activation. To test this hypothesis, proteasome function in HUVECs was inhibited with the peptide aldehydes MG 115 or N-acetyl-leucine-leucine-not leucine (ALLN).14,15 As shown in Figure 3A, MG 115 (30 μmol/L) or pervanadate (200 μmol/L) did not affect, however, on NF-κB activation in response to oxidative stress in HUVECs treated with pervanadate. Identical results were obtained with ALLN (data not shown; 4 separate experiments). These results further support the possibility that oxidative stress-induced NF-κB translocation to the nucleus can occur independently of IκB degradation, suggesting that alternate pathways of NF-κB activation exist in cardiovascular cells sustaining I/R injury.

**Discussion**

Endothelial cell activation is mediated through NF-κB in response to diverse stimuli. It is unknown, however, whether each stimulus activates NF-κB through the same signaling pathway or through different and unique pathways. Our findings indicate that in HUVECs, oxidative stress activates NF-κB through a mechanism that does not require IκBα degradation, a biochemical event that is required for TNF-α-induced NF-κB activation. IκBβ or IκBε degradation, as measured by Western blot analysis with polyclonal antibodies specific for each inhibitor protein, was not observed in HUVECs exposed to oxidative stress (data not shown). Although not degraded, IκB is most likely modified in some fashion during oxidative stress to cause IκB to dissociate from NF-κB, exposing a nuclear localization signal in NF-κB as a prerequisite to nuclear translocation of NF-κB. This conjecture, however, was not examined in the present study.

TNF-α stimulation of HUVECs results in rapid phosphorylation of IκBα serine residues 32 and 36 by a multimeric IκB kinase complex, which is activated by other upstream kinases.7,16 Serine phosphorylation of IκBα is followed by addition of a ubiquitin protein to IκBα and degradation in the proteasome. Recent reports have demonstrated that IκB can also be phosphorylated on a tyrosine residue at position 42 of IκBα, which is in close proximity to the 2 serine phosphoac-
ceptor sites. Tyrosine-phosphorylated IκBα is protected from TNF-α–induced degradation, although the mechanism of this protective effect is not known. In Jurkat T-cells, tyrosine phosphorylation of IκBα is observed after exposure of this cell line to pervanadate or to hypoxia followed by reoxygenation, and tyrosine phosphorylation produces dissociation of tyrosine-phosphorylated IκBα from NF-κB. Tyrosine phosphorylation of IκBα in Jurkat T-cells after exposure to hypoxia only has been observed as well.19 In the study with hypoxic Jurkat T-cells, IκBα degradation was observed 60 minutes after the onset of hypoxia. In contrast, we did not observe IκBα degradation in HUVECs after 60 minutes of hypoxia. Moreover, NF-κB activation in hypoxic HUVECs occurs within 15 minutes of the onset of reoxygenation of hypoxic cells. Therefore, we are unable to relate the response occurs within 15 minutes of the onset of reoxygenation of hypoxia.

The results we obtained in vitro may be relevant to I/R injury in vivo. In patients undergoing open heart surgery, we assayed right atrial tissue samples for NF-κB activation. Tissue was obtained before CPB and 45 minutes after CPB was discontinued. We showed that NF-κB is activated in human hearts reperfused for 45 minutes after the discontinuation of CPB, but not in skeletal muscle samples that were continuously perfused and oxygenated. This is the first demonstration of NF-κB in vivo in human hearts with I/R injury.

In summary, our results suggest that oxidative stress in human vascular cells may activate the proinflammatory transcription factor NF-κB through an alternative pathway independent of IκBα degradation. Thus, the inflammatory response to oxidative stress involving NF-κB and occurring during I/R injury may be inhibited without compromising a cytokine-induced, NF-κB–mediated inflammatory response to infection.

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References
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