

NF- κ B Activation Plays a Role in Superoxide-Mediated Cerebral Endothelial Dysfunction After Hypoxia/Reoxygenation

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Background and Purpose—Cerebral vascular injury occurs in response to hypoxia/reoxygenation (H/R). However, the cellular signaling pathways that regulate this event remain unclear. The present study was designed to determine whether reactive oxygen species (ROS) mediate endothelial dysfunction after H/R in cerebral resistance arteries and, if so, the relative contribution of ROS, NADPH oxidase, and a nuclear factor- κ B (NF- κ B) pathway.

Methods—Arterial diameter and intraluminal pressure were simultaneously measured on rat posterior cerebral arteries (PCA). Superoxide was measured by 5- μ mol/L lucigenin-enhanced chemiluminescence.

Results—Hypoxia/reoxygenation selectively inhibited cerebral vasodilation to the endothelium-dependent agonist acetylcholine (ACh) (0.01 to 10 μ mol/L) by \approx 50%. Impaired vasodilation after H/R was reversed by 2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempo) (100 μ mol/L), a cell-permeable superoxide dismutase mimetic, and partially by ebselen (10 μ mol/L), a peroxynitrite scavenger. H/R-impaired vasodilation to ACh was also preserved by apocynin (1 mmol/L), a specific inhibitor for NADPH oxidase. Correspondingly, H/R significantly increased lucigenin-detectable superoxide, which was reduced by either Tempo or apocynin, but not by allopurinol (10 μ mol/L), an inhibitor of xanthine oxidase. Finally, the NF- κ B inhibitors helenalin (10 μ mol/L) and MG-132 (1 μ mol/L) independently antagonized H/R-impaired ACh-induced vasodilation without affecting dilator response to sodium nitroprusside, an endothelium-independent vasodilator.

Conclusions—These results indicate that superoxide mediates cerebral endothelial dysfunction after hypoxia/reoxygenation largely via activation of NADPH oxidase and possibly activation of NF- κ B pathway. (*Stroke*. 2005;36:1047-1052.)

Key Words: cerebral arteries ■ endothelium ■ hypoxia ■ superoxide

Hypoxia and reoxygenation are 2 essential elements of ischemia/reperfusion injury. Hypoxia/reoxygenation (H/R) may cause different forms of vascular injury, such as hemorrhage, changes in vascular permeability, and endothelial dysfunction, including impaired endothelium-dependent vasodilation.¹⁻⁶ H/R-impaired endothelium-dependent vasodilation has been demonstrated in blood vessels from peripheral circulation⁴⁻⁸ and cerebral circulation.⁹⁻¹⁰

A pivotal feature of response to H/R is the generation of reactive oxygen species (ROS) by a diversity of cell types, including endothelial cells. Numerous studies suggest that ROS are central mediators of cellular injury that occurs after ischemia/reperfusion.^{11,12} Furthermore, endothelial dysfunction characterized by impaired endothelium-dependent vasodilation has been attributed to increased inactivation of nitric oxide (NO) by superoxide anion (O₂⁻) rather than impaired generation of NO in various disease models,¹³ such as hypertension^{14,15} and atherosclerosis.¹⁶ It has been demonstrated that the formation of ROS after ischemia/reperfusion

occurs in the brain and affects cerebral vascular tone.^{9,10,17-19} However, cellular mechanisms underlying H/R-induced cerebral vascular injury have not been well-defined.

The nuclear factor- κ B (NF- κ B) plays a key role in the regulation of cellular responses to tissue injury. Although it has been reported that H/R-induced vascular injury is associated with the activation of NF- κ B pathway,^{20,21} there is no direct evidence regarding how this association contributes to the regulation of cerebral vascular tone.

The objectives of the present study were to determine whether ROS mediate endothelial dysfunction after H/R in cerebral resistance arteries and, if so, the relative contribution of ROS, NADPH oxidase, and NF- κ B pathway. Because endothelial dysfunction is a significant event in virtually all forms of ischemia/reperfusion injury,^{22,23} this element was chosen for our focus; we evaluated it based on vascular responses to acetylcholine (ACh), an endothelium-dependent agonist that produces NO-dependent vasodilation of cerebral circulation.²⁴

Received December 1, 2004; accepted January 18, 2005.

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Stroke is available at <http://www.strokeaha.org>

DOI: 10.1161/01.STR.0000157664.34308.cc

Materials and Methods

General Preparations

Male Sprague-Dawley rats (105 rats, weight 250 to 300 grams, from Taconic, Germantown, NY) were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) along with heparin (1000 U/kg intraperitoneally) before exsanguination. The brain was rapidly removed and placed in ice-cold physiological salt solution (PSS) with the following composition (in mmol/L): NaCl, 119; KCl, 4.7; CaCl₂, 1.6; MgSO₄, 1.17; KH₂PO₄, 1.18; NaHCO₃, 24.9; EDTA, 0.026; and dextrose, 5.5; pH was 7.4. The posterior cerebral artery (PCA) was carefully isolated. All procedures were approved by the Institutional Animal Care and Use Committee at Children's National Medical Center.

Measurement of Vascular Diameter

As previously described,^{25,26} segments of the PCA (1 to 2 mm in length, 120±10 μm in internal diameter) were placed in a microvascular chamber containing PSS aerated with 16% O₂/5% CO₂/79% N₂ (pH=7.4) at room temperature. Vessels were cannulated with glass pipettes and pressurized to 60 mm Hg after making sure there were no potential leaks. The cannulated vessel was warmed to 37°C and allowed to equilibrate for 30 to 60 minutes under no flow condition, with a longitudinal stretch to approximate its in situ length, until stable myogenic tone developed. The blood vessel was imaged using a video camera and a dimension analyzer (Living Systems Instrumentation) linked to a chart recorder (Model 23; Perkin-Elmer). Inner diameter and intravascular pressure were measured continuously throughout the experiment. All pharmacological reagents were added to the superfusion solution. At the end of each experiment, the diameter of maximal dilation was determined in the presence of Ca²⁺-free medium with EGTA (2 mmol/L).

Experimental Protocols: Effects of In Vitro H/R

H/R was accomplished by changing the superfusate pO₂ in an airtight arteriograph reservoir system.^{7,27} After vascular reactivity was stabilized, the vessels were exposed to hypoxia (95% N₂/5% CO₂, superfusate pO₂ 10 to 15 mm Hg) for 30 minutes and then to reoxygenation (16% O₂/5% CO₂/79% N₂, superfusate pO₂ 100 to 160 mm Hg) for 30 minutes. Concentration-response curves to Ach (0.01 to 10 μmol/L) on a 5-hydroxytryptamine-constricted vessel were constructed, and responses to sodium nitroprusside (SNP) (5 μmol/L) and KCl (60 mmol/L) were compared before and after H/R.

Cellular Mechanisms

The effect of H/R on cerebral vasodilation to Ach was examined in the absence and presence of 60-minute treatment with 2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempo) (100 μmol/L), a cell-permeable superoxide dismutase (SOD) mimetic,^{14,28} catalase (200 U/mL), a scavenger of hydrogen peroxide (H₂O₂), deferoxamine (100 μmol/L), a scavenger of hydroxyl radical (OH⁻), ebselen (10 μmol/L), a scavenger of peroxynitrite (ONOO⁻),²⁹ indomethacin (10 μmol/L), a cyclooxygenase inhibitor, apocynin (1 mmol/L), a specific inhibitor of NADPH oxidase,³⁰ and 2 inhibitors of NF-κB activation: helenalin (10 μmol/L)³¹ and Z-Leu-Leu-Leu-CHO (MG-132; 1 μmol/L).³² Effects of selective inhibitors on SNP-induced dilation were compared. Vascular responses to pyrogallol (100 μmol/L; an O₂⁻ generator) and exogenous H₂O₂ (1 μmol/L) were also examined to verify the specific effect of Tempo. Vehicle control experiments showed that vasodilation was unaffected with DMSO at a maximal final concentration of <0.1%.

Measurement of Vascular O₂⁻

Superoxide was measured by lucigenin-enhanced chemiluminescence.³³ Briefly, cerebral resistance arteries were prepared as described for arteriograph studies, except that pooled arteries were used to ensure a detectable signal. The arteries were placed in a scintillation vial containing lucigenin (5 μmol/L) and other additions in a

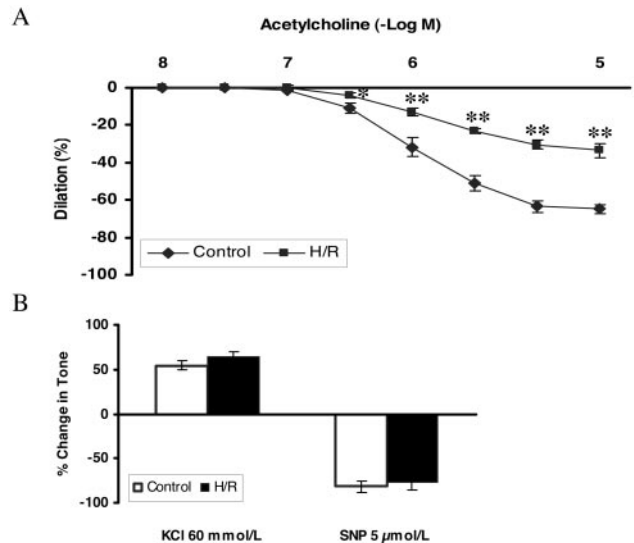


Figure 1. Effects of H/R on responses of rat posterior cerebral arteries (PCA) to (A) acetylcholine (Ach; n=10), and (B) KCl (n=5) and sodium nitroprusside (SNP; n=5). Values are mean±SE. **P*<0.05, ***P*<0.01 vs control.

final volume of 1 mL PSS (pH=7.4). The O₂⁻-induced chemiluminescence was measured in a liquid scintillation counter (Beckman LS6500). All manipulations were performed in a light-protected environment. After a 5-minute dark adaptation, samples were counted once for 0.1 minute each and then recounted over the next 30 minutes. NADPH (100 μmol/L) was used to stimulate O₂⁻ level under conditions other than the basal level. In pilot experiments, the measurement was validated by using a NAD(P)H oxidase inhibitor diphenylene iodonium (10 μmol/L) and its solvent. All treatments were conducted at 37°C in the same manner as described above. The background was subtracted to obtain the data reported as counts·min⁻¹·mg protein⁻¹. Protein was measured using the Bradford method.

Data Analysis

All data are presented as mean±SE, and n indicates the number of animals for the experiments performed. Vasodilation is expressed as percent change of steady level of precontraction (ie, percent of 5-hydroxytryptamine-induced tone). Data were analyzed by the paired Student *t* test for single comparisons and by 1-way ANOVA for multiple comparisons, followed by Bonferroni test when appropriate. Differences were considered significant when *P*<0.05.

Results

H/R Impaired Endothelial Function

Under control conditions, baseline diameter of rat PCA averaged 146±3 μm, and fully distended diameter (ie, in Ca²⁺-free medium with 2 mmol/L EGTA) averaged 223±3 μm (n=50). Approximately 98% of the rat PCA segments developed myogenic tone with PSS (37°C) within 1 hour at 60 mm Hg intramural pressure. Although hypoxia induced vasodilation (154±4 μm) initially, vessel diameters at the end of hypoxia (144±4 μm) and reoxygenation (142±5 μm) were not different from the control (146±5 μm; n=30; *P*=0.6).

Ach initiated reproducible dilation of precontracted PCA in a concentration-dependent manner. Dilation to Ach was significantly attenuated by ≈50% at concentrations of 3 to 10 μmol/L after H/R (Figure 1A). In contrast, dilation

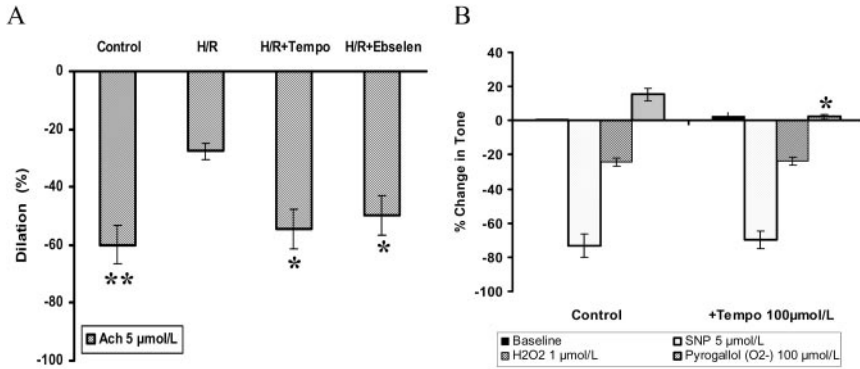


Figure 2. Effect of Tempo. A, Dilator response of rat PCA to Ach (5 μ mol/L) after H/R in the absence and presence of Tempo (100 μ mol/L) and ebselen (10 μ mol/L). Values are mean \pm SE, n=5 to 10. * P <0.05, ** P <0.01 compared with H/R. B, Specificity of O₂⁻-scavenging effect of Tempo (n=4 to 8). Values are mean \pm SE, * P <0.05 vs control.

induced by SNP (5 μ mol/L) and constriction to KCl (60 mmol/L) were not altered by H/R (Figure 1B). In time-control experiments, diameters of PCAs did not change during continuous exposure to PSS with 16% O₂/5% CO₂/79% N₂, demonstrating that the baseline did not drift with time. These results indicated that H/R caused selective inhibition of endothelium-dependent response to Ach in the cerebral arteries.

Cellular Mechanisms: Superoxide Mediated H/R-Impaired Endothelial Function

Tempo (100 μ mol/L), the cell-permeable O₂⁻ scavenger, almost reversed the inhibitory effect of H/R on Ach-induced dilation (Figure 2A), but had no effect on Ach-induced dilation in the absence of H/R (data not shown). Specificity of Tempo as O₂⁻ scavenger is shown in Figure 2B. These data indicated that superoxide production was responsible for H/R-impaired vasodilation.

Ebselen (10 μ mol/L), the ONOO⁻ scavenger, partially preserved Ach-induced dilation after H/R (Figure 2A) and also modified baseline diameter (144 \pm 5 versus 163 \pm 4 μ m; P <0.05). H/R-impaired Ach-induced dilation (30.0 \pm 3.4%) was not significantly influenced by either catalase (200 U/mL; 48.3 \pm 6.7%) or deferoxamine (100 μ mol/L, 45.4 \pm 7.6%) (n=5 per group; P >0.05). Apocynin (1 mmol/L), the specific inhibitor of NADPH oxidase, also significantly preserved Ach-induced dilation after H/R (Figure 3) and had no significant effect on baseline diameter (145 \pm 6 versus 136 \pm 5 μ m). Indomethacin (10 μ mol/L) was ineffective (42.4 \pm 15.2%; n=5; P >0.05).

NF- κ B Participated in H/R-Impaired Endothelial Function

MG-132 (1 μ mol/L) significantly antagonized the inhibitory effect of H/R on Ach-induced dilation, as did helenalin (10 μ mol/L) (Figure 4). In contrast to Ach-induced dilation, SNP-induced dilation was unaffected by these inhibitors (Figure 5). Baseline diameters were not significantly altered by MG-132 (148 \pm 8 versus 142 \pm 8 μ m) and helenalin (138 \pm 5 versus 141 \pm 10 μ m). The NF- κ B inhibitors had no effect on Ach-induced dilation in the absence of H/R (data not shown).

Superoxide Level Increased After H/R

Basal levels of superoxide in these arteries were relatively low (12.89 \pm 3.4 cpm/mg protein). Lucigenin-detectable O₂⁻ signal was remarkably increased in the presence of NADPH (100 μ mol/L). H/R further increased O₂⁻ level by >2.5-fold (P <0.05). Tempo and apocynin, but not allopurinol, effectively inhibited O₂⁻ levels to 15% to 16%. Data are summarized in Figure 6.

Discussion

The major findings in the present study include the following: (1) vascular superoxide is responsible for the selective impairment of endothelium-dependent dilation after H/R in isolated cerebral arteries; (2) apocynin-sensitive NADPH oxidase in the cerebral vascular wall is a potential source of superoxide production; and (3) our data are consistent with activation of NF- κ B playing a role in H/R-induced cerebral endothelial dysfunction. To our best knowledge, this is the first study to document that NF- κ B activation in response to H/R may contribute to functional regulation of resistance arteries from cerebral circulation.

It is likely that intracellular O₂⁻ is the primary mediator of H/R-induced cerebral endothelial dysfunction. Endothelial NADPH oxidase increases superoxide intracellularly.^{34,35} Furthermore, superoxide is charged and may not easily diffuse across cell membranes. Exogenous SOD enzyme activity is primarily extracellular, whereas the cell-permeable SOD mimetic Tempo is capable of scavenging superoxide from both intracellular and extracellular environments.^{14,28} In this study, we found that Tempo almost completely reversed H/R-mediated impairment of Ach-induced vasodilation and remarkably suppressed H/R-induced superoxide production. The effect of Tempo seems to be selective, because it has no

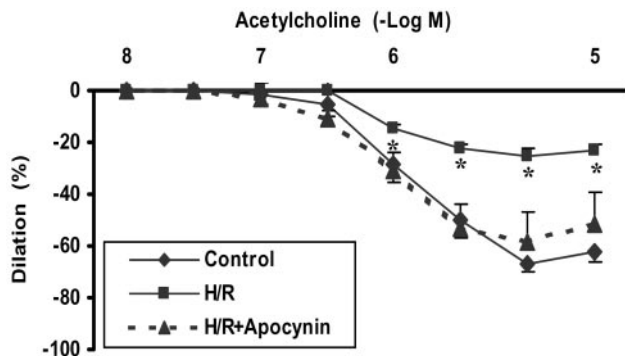


Figure 3. Effect of apocynin (1 mmol/L) on H/R-impaired dilation of rat PCA to Ach. Values are mean \pm SE, n=5. * P <0.05 vs control and H/R plus apocynin.

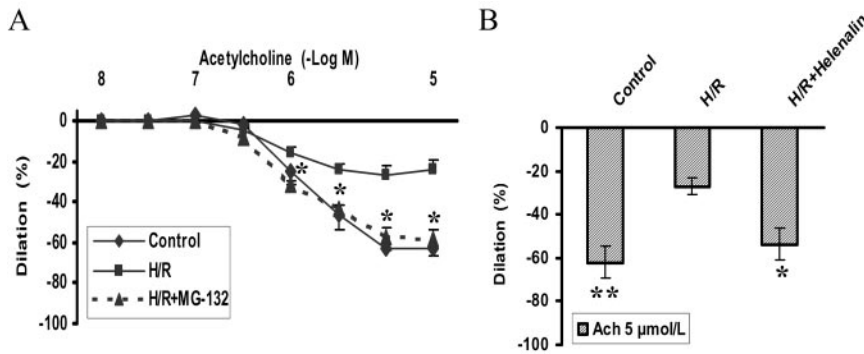


Figure 4. Dilatation of rat PCA to Ach after H/R in the absence and presence of NF-κB inhibitors. A, Effect of MG-132 (1 μmol/L, n=4). B, Effect of helenalin (10 μmol/L, n=7). Values are mean±SE. *P<0.05, **P<0.01 vs H/R.

influence on basal tone, SNP-induced endothelium-independent dilation, and/or exogenous H₂O₂-induced vasodilation, yet it inhibits O₂⁻-induced vasoconstriction and O₂⁻ levels. It is conceivable that O₂⁻ might not be the only free radical generated in response to H/R, given the quick and sequential reactions among ROS. After all, because O₂⁻ is the precursor of H₂O₂, which ultimately produces OH⁻, the removal of O₂⁻ might reduce the formation of other ROS that could contribute to H/R-induced vascular injury. Our results confirm and extend previous studies showing that SOD-inhibitable superoxide production plays a key role in cerebral endothelial dysfunction after H/R.^{10,17}

It appears that peroxynitrite (ONOO⁻) might be the secondary mediator responsible for impaired vasodilation to Ach after H/R. This is based on the data that ebselen, the membrane-permeable ONOO⁻ scavenger,²⁹ substantially preserved dilator response to H/R, suggesting that ONOO⁻ is a participant in H/R-induced endothelial dysfunction. Similarly, a recent report showed that ONOO⁻ is involved in reperfusion-induced cerebral vascular injury.³⁶ Thus, an alternative explanation of our findings is that superoxide generated by vascular NADPH oxidase, with subsequent formation of ONOO⁻, mediates cerebral endothelial dysfunction. Although H₂O₂ and OH⁻ take part in alteration of vascular function in other cerebral vessels,^{10,37,38} our data with catalase and deferoxamine suggest that these radicals are not critical for H/R-impaired vasodilation in this model. These divergent results may be largely caused by using different animals or experimental models, the complexity of regulatory mechanisms involving ROS, and the heterogeneity of vascular endothelium.

The involvement of NADPH oxidase in cerebral endothelial response to H/R is evident. A major source of vascular production of ROS is the NADPH oxidases.^{39,40} Apocynin, the specific inhibitor for NADPH oxidase, has been shown to prevent the binding of cytosolic subunits of NADPH oxidase to membrane-bound p22^{phox}/gp91^{phox} subunits, thereby inhibiting oxidase activation and subsequent production of superoxide.³⁰ Thus, the effect of apocynin is more specific than that of diphenylene iodonium, the inhibitor of flavin-containing proteins. Our data show that apocynin may significantly protect H/R-impaired vasodilation and suppress H/R-increased superoxide. This is in agreement with previous findings that NADPH oxidase activity is the potential source of superoxide and responsible for the regulation of cerebral vascular tone.⁴¹ Furthermore, a preventive effect of apocynin on ischemia/reperfusion-induced pulmonary vascular injury has been demonstrated.³ Interestingly, the NADPH oxidase subunit p47phox participates in the activation of NF-κB in endothelial cells.⁴² Although superoxide generation may be facilitated by xanthine oxidase, cyclooxygenase, and endothelial NO synthase, these enzymes are probably less involved in endothelial dysfunction observed in this study because the inhibitors of these enzymes failed to modify H/R-mediated superoxide production and vasodilation.

Our pharmacological interventions of NF-κB pathway antagonized impaired endothelial dilator response to H/R in the following ways. First, helenalin inhibits NF-κB by spe-

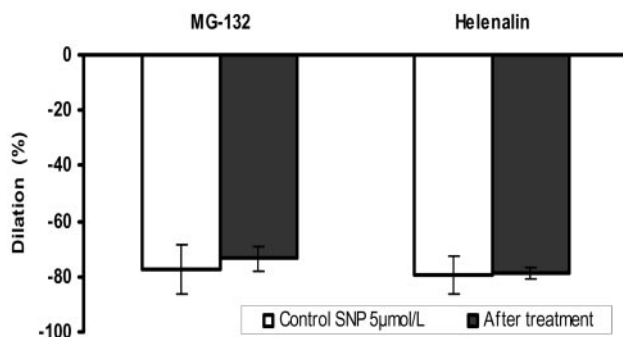


Figure 5. Effect of MG-132 (1 μmol/L) and helenalin (10 μmol/L) on dilator response of rat PCA to sodium nitroprusside (SNP; 5 μmol/L). Values are mean±SE, n=5.

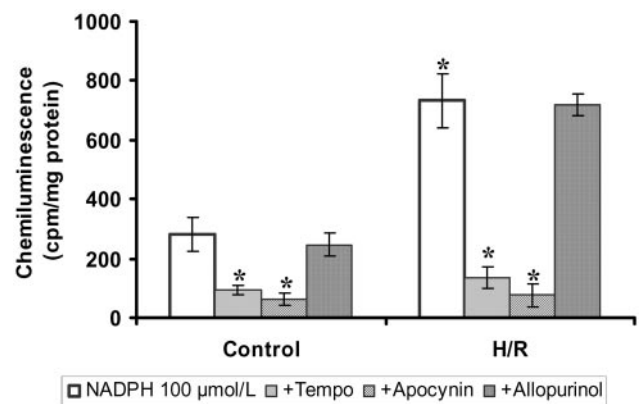


Figure 6. Levels of superoxide measured by lucigenin-enhanced chemiluminescence in control and H/R-treated rat cerebral arteries in the absence and presence of Tempo (1 mmol/L), apocynin (1 mmol/L), and allopurinol (10 μmol/L). Values are mean±SE, n=4 to 6. *P<0.05 vs the respective control.

cific and irreversible alkylation of the p65 subunit, thereby blocking DNA binding.³¹ Second, MG-132, the potent and selective proteasome inhibitor, inhibits NF- κ B activation by preventing degradation of an inhibitory protein called I- κ B.³² Finally, SN50 inhibitory peptide, by inhibiting the translocation of NF- κ B active complex into the nucleus,⁴³ protected endothelial dysfunction after H/R (Xie et al, unpublished data, 2004). Because these inhibitors exert the same protective effect by acting on different sites of NF- κ B pathway, it is unlikely that this effect is a result of nonspecific alteration of vascular tone. Our results with isolated cerebral arteries are supported by the findings obtained from cultured cerebral endothelial cells.^{20,44} Although time of NF- κ B activation may vary from minutes to hours or slower depending on cell types and stimuli, the time course of this study is consistent with that demonstrated in cultured cerebral endothelial cells, in which activation of NF- κ B occurs as early as 15 to 30 minutes after H/R.²⁰ We are aware that no direct measurement of NF- κ B activation is a limitation of this study.

Several important questions remain unanswered, including how NF- κ B is activated, how activation of NF- κ B quickly alters vascular function by altering gene expression, and what are its target genes. It can be speculated that NF- κ B is activated after exposure to H/R, with this activation inducing the transcription of genes that synthesize small soluble cytoplasmic proteins within 30 to 60 minutes. These proteins could act via a Ca²⁺-dependent pathway or ion channels to modify vascular function. Therefore, the possibility that NF- κ B-inducible proteins disclose or auto-amplify other signaling pathways cannot be excluded. In addition, unidentified factors might act earlier than changes in gene expression.

H/R-induced vascular injury is of significance in cardiovascular pathophysiology because it occurs in a wide variety of clinical conditions, such as myocardial infarction, stroke, shock, and organ transplantation, as well as in infants with respiratory failure subjected to extracorporeal membrane oxygenation.⁴⁵ It can be argued that experimental H/R may not accurately represent what happens in vivo. However, it is important to stress that endothelial dysfunction manifested during H/R in vitro is consistent with the susceptibility of brain microvessels to ischemia/reperfusion observed in vivo.

In summary, our findings indicate that H/R-induced cerebral endothelial dysfunction is mediated by NADPH oxidase-generated superoxide and is possibly associated with NF- κ B-dependent pathway. Therefore, this study may provide potential targets to help development of therapeutic strategies for ischemia/reperfusion injury, particularly in cerebral circulation.

Acknowledgments

This work was supported by grant NS-40381 from the National Institutes of Health.

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