Reperfusion-Induced Oxidative/Nitrative Injury to Neurovascular Unit After Focal Cerebral Ischemia

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Background and Purpose—Use of thrombolysis in stroke is limited by a short therapeutic window because delayed reperfusion may cause brain hemorrhage and edema. Available evidence suggests a role for superoxide, NO, and peroxynitrite in reperfusion-induced injury. However, depending on their cellular origin and interactions between them, these molecules may exert protective or deleterious actions, neither of which is characterized in the intact brain.

Methods—Using fluorescent probes, we determined superoxide and peroxynitrite formation within neurons, astrocytes, and endothelium, and the association between oxidative/nitrative stress and vascular injury in mice brains subjected to 2-hour middle cerebral artery occlusion and 3 or 5 hours of reperfusion.

Results—Both signals were colocalized, suggesting that the main source of peroxynitrite in the reperfused brain was a reaction between superoxide and NO. Superoxide and peroxynitrite formation was particularly intense in microvessels and astrocytic end-feet surrounding them, and overlapped with dense mitochondrial labeling. Sites of oxidative/nitrative stress on microvessels were colocalized with markers of vascular injury such as Evans blue (EB) leakage and matrix metalloproteinase-9 (MMP-9) expression, suggesting an association between peroxynitrite and microvascular injury. Supporting this idea, partial inhibition of endothelial NO synthesis at reperfusion with a low dose of L-nitroarginine (1 mg/kg IP) reduced 3-nitrotyrosine formation in microvessels and EB extravasation.

Conclusion—During reperfusion, intense superoxide, NO, and peroxynitrite formation on microvessels and surrounding end-feet may lead to cerebral hemorrhage and edema by disrupting microvascular integrity. Combination of thrombolysis with agents diminishing oxidative/nitrative stress may reduce reperfusion-induced injury and extend the therapeutic window for thrombolysis. **(*Stroke*. 2004;35:1449-1453.)**

Key Words: blood-brain barrier ■ matrix metalloproteinases ■ nitric oxide ■ peroxynitrite ■ reactive oxygen species ■ reperfusion injury ■ thrombolysis

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Thrombolytic therapy cannot be administered to most stroke patients arriving at the hospital several hours after stroke because delayed recirculation bears risk of brain hemorrhage and edema.[1] An increased production of superoxide and NO in the brain has been demonstrated after reperfusion.[2,3] A concomitant surge in production of these radicals can lead to formation of peroxynitrite, a powerful oxidant.[4,5] Overproduction of these radicals may play important roles in reperfusion-induced injury. Supporting this view, scavengers of free radicals or inhibitors of lipid peroxidation protect the brain after ischemia/reperfusion.[6,7] Mice overexpressing superoxide dismutase (SOD) are more resistant to ischemia associated with reperfusion,[8] and conversely, larger infarcts develop in SOD knockout mice.[9,10] NO's role is more complex since it has both beneficial and deleterious actions during ischemia/reperfusion depending on the cell type in which it is generated.[11] Excessive NO generation by nNOS is cytotoxic and, accordingly, nNOS knockouts are resistant to ischemia.[12] On the contrary, eNOS knockouts develop larger infarcts because NO of endothelial origin promotes survival by improving residual blood flow during ischemia.[13] However, the role of eNOS is more complicated because endothelial NO surge during reperfusion may contribute to brain injury via peroxynitrite formation, on one hand,[14] and may improve microcirculation by preventing platelet and neutrophil aggregation, on the other.[15]

Consequently, there is controversy about the roles of superoxide, NO, and peroxynitrite in reperfusion injury because in vivo interactions between them are not characterized, and they may exert opposing actions depending on their cellular origin, distance between their sources, and in vivo diffusibility.[5,11] It has been proposed that superoxide is not a powerful oxidant but gains significant toxicity by being converted to peroxynitrite after interacting with NO.[5] Conversely, peroxynitrite is thought by some authors not to reach toxic concentrations during reperfusion but exert a favorable action by reducing endothelial dysfunction and leukocyte adhesion.[16] Therefore, we have investigated the cellular

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origins of these radicals and the spatial relationship between them in reperfused brain in vivo.

Methods

Middle Cerebral Artery Occlusion

Animal housing, care, and application of experimental procedures were done in accordance with institutional guidelines. Swiss albino mice (28 to 34g) were fasted overnight with free access to water before the experiment, and were anesthetized with chloral hydrate (400 mg/kg, IP). Body temperature was maintained at 37.0±0.1°C during the experiment and only animals with arterial blood pressure in physiological ranges were included in the study. Proximal occlusion of the right middle cerebral artery (MCA) was performed with a filament as described before.[14] The regional cerebral blood flow (rCBF) was monitored by laser-Doppler flowmetry with a probe placed over the skull to ensure that a desired level of ischemia and reperfusion was achieved. Forty mice were subjected to 2 hours of proximal MCA occlusion and 3 (n=34) or 5 (n=6) hours of reperfusion.

Detection of Superoxide

Dihydroethidium (HEt, Molecular Probes, 2 mg/200 μL PBS) was given intravenously just before reperfusion to monitor superoxide production.[9,17] Hydroethidine (HEt) is oxidized by superoxide to ethidium and an unidentified product (Et+X), which can be detected with fluorescent microscopy (Ex=543 nm and Em>570 nm).[9,17,18] This red fluorescence has been repeatedly shown to be inhibited by SOD and superoxide scavengers in heart and brain, and is used for semiquantitative intracellular superoxide detection.[18] After 3 or 5 hours of reperfusion, mice were perfused transcardially with heparinized saline followed by 10% formaldehyde. Brains were postfixed in 10% formaldehyde for 24 hours and then were cryoprotected in 20% and 30% sucrose. Fifteen-μm–thick sections passing through the anterior commissure were kept frozen at −20°C until use. Sections were washed 3 times in a blocking and aldehyde-reducing solution.

Immunohistochemistry

We monitored peroxynitrite formation by detecting nitrosylated tyrosine residues on proteins with anti-3–nitrotyrosine (3-NT) antibody (Upstate Biotechnology). Neurons, astrocytes, and endothelial cells were labeled with antibodies against microtubule-associated protein-2 (MAP-2; Sigma), glial fibrillary acidic protein (GFAP; Sigma), and endothelium (CD-34; Chemicon), respectively. Matrix metalloproteinase-9 (MMP-9) expression was detected with anti-MMP-9 antibody (Chemicon). Mitochondria were labeled with antiprohibitin antibody (Research Diagnostics) or Mitotracker (Molecular Probes). Sections were incubated first with primary antibodies (1:100) at 37°C for 90 minutes and then with secondary antibodies (1:200) for 90 minutes. Fluorescein isothiocyanate (FITC) goat anti-mouse IgG, Cy2 goat anti-rabbit IgG, Cy3 goat anti-rabbit IgG, and Cy3 goat anti-mouse IgG (all from Jackson ImmunoResearch) were used as appropriate. Primary antibody omission incubations with either blocking solution or PBS were performed to test the specificity of immunoreactivity.

Sections were mounted in glycerol/PBS medium containing 25 mg/mL sodium azide. Specimens were examined by a Zeiss LSM-510 confocal laser-scanning microscope. Single optical sections (2048×2048 pixel) were collected. Digitized images were pseudocolored according to their original fluorochromes.

Colocalization of 3-Nitrotyrosine With Evans Blue Extravasation and MMP-9

Breakdown of blood-brain barrier (BBB) was assessed by Evans blue (EB) extravasation, which fluoresces red when excited. EB (0.3 mL of 4% solution; Sigma) was injected through the tail vein before reperfusion. Three or 5 hours after reperfusion, mice were transcardially perfused and frozen sections were obtained as described under Detection of Superoxide. The sections were double-labeled with MMP-9 or 3-NT antibody and colocalization of signals was assessed with confocal microscopy.

Fluorometric Measurement of Evans Blue Leakage

EB extravasation was quantified in L-nitroarginine (L-NA) and vehicle-treated mice 3 hours after reperfusion (n=6 per group). Transcardially perfused brains were removed and each hemisphere was homogenized in 1 mL 50% trichloroacetic acid and then centrifuged at 14 000*g* for 20 minutes at 4°C.[14] The supernatant was diluted 4-fold with ethanol. Fluorescence intensity was measured by a microplate reader (620/680 nm; Wallac; Perkin–Elmer). Calculations were based on external standard readings and extravasated EB dye was expressed as micrograms/per hemisphere.

Cell Count and Statistics

Superoxide or 3-NT–positive cells were manually counted over the ischemic hemispheres in 5 selected core regions (frontal, parietal, insular cortices, preoptic area, and lateral striatum) under ×400 magnification. Ratios of those positively-labeled to the total number of neurons, astrocytes, or endothelia in each microscopic field were individually calculated. Cell counts were also obtained from another group of mice subjected to 2 hours ischemia and 3 hours reperfusion, but treated with L-NA (1 mg/kg, IP) or 7-nitroindazole (7-NI) (50 mg/kg, IP) before reperfusion. Mean values are given with their standard deviations, and the number of animals and brain sections studied are indicated in parentheses under Results. Groups were compared by analysis of variance (ANOVA); $P<0.05$ was considered to be significant.

Results

Superoxide and Peroxynitrite Formation After Reperfusion

In the nonischemic hemisphere, there were only occasional 3-NT–positive cells, whereas cells generating superoxide were more common in agreement with previous reports.[9,17] On the other hand, numerous cells in the ischemic MCA territory were strongly labeled with 3-NT (n=4 mice, 16 sections) and Et+X (n=4,12), 3 hours after reperfusion (Figure 1). The size of the Et+X (n=2.8) or 3-NT (n=2.6) positive granules increased when reperfusion was extended to 5 hours. Both signals were more prominent in ischemic core. Double-labeling studies with cell-specific markers showed that 3-NT and Et+X fluorescence were scattered in neurons, astrocytes, and endothelial cells (Figure 1). Most neurons (79±18% of all MAP-2-positive cells; n=4,12) were labeled with anti-3–NT antibody (Figures 1 and 2) and displayed Et+X fluorescence (78±8%; n=4,12). Only half of the astrocytes labeled with GFAP displayed 3-NT immunolabeling (51±10%; n=4,12) and Et+X fluorescence (47±10%; n=4,12). 3-NT and Et+X positivity were dispersed in cell bodies as well as processes of neurons and astrocytes. All Et+X signals were colocalized with 3-NT signals (weighted colocalization coefficient; 0.89±0.03), except the small 3-NT granular staining scattered in neuropil (Figure 1). Accordingly, the number of Et+X- and 3-NT–labeled cells were similar for all 3 cell types (Figure 2). Consistent with our previous report showing a striking 3-NT staining (diaminobenzidine-labeled) of microvessels relative to parenchyma,[14] fluorescent markers disclosed that astrocytic endfeet ensheathing microvessels and endothelia were intensely positive for both signals (Figure 1). 48±17% of CD-34–positive endothelial cells were labeled with anti-3–NT antibody (n=4,12) and displayed Et+X fluorescence (51±13%, n=4,12) (Figure 2). The area of intense Et+X as well as 3-NT fluorescence on microvessels overlapped with dense mitochondrial labeling observed with 2 different markers of mitochondria (n=3,6) (Figure 3B and 3C).

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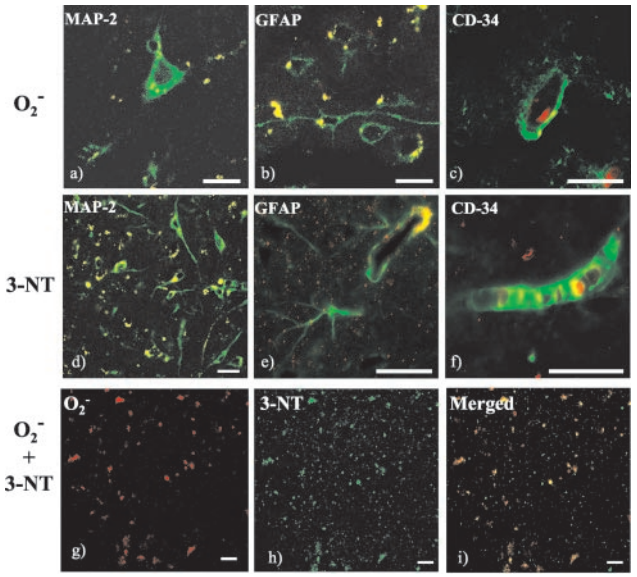

Figure 1. Focal ischemia/reperfusion induces superoxide and peroxynitrite formation in neurons (MAP-2), astrocytes (GFAP), and endothelium (CD-34). Three hours after reperfusion, superoxide (red dihydroethidium fluorescence) and 3-NT immunofluorescence (red) increased in all cell types (green). Endothelia (c, f) and astrocytic end-feet ensheathing microvessels (e, arrow) were intensely labeled. Note superoxide leakage into lumen in c. Superoxide and peroxynitrite signals were colocalized (yellow) with 3-NT labeling, except small granular 3-NT stainings in neuropil (g, h, i). Scale bars=20 μm.

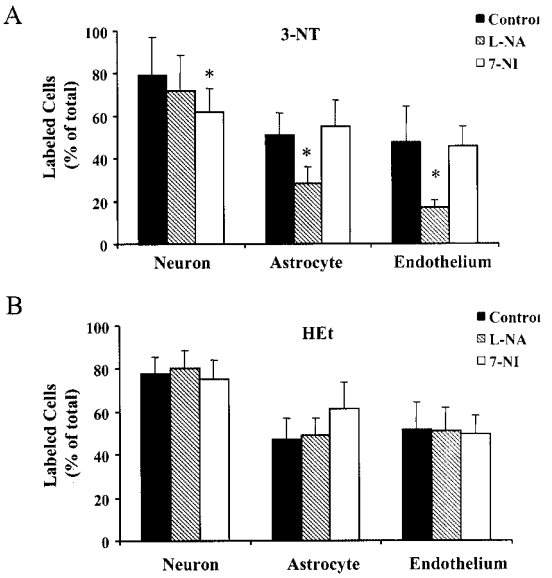

Figure 2. A, L-NA (1 mg/kg, IP) administered 15 minutes before reperfusion decreased peroxynitrite formation (3-NT immunoreactivity) in endothelia and astrocytes. 7-nitroindazole (7-NI) (50 mg/kg, IP) given 30 minutes before reperfusion reduced neuronal but not endothelial 3-NT-labeling. B, Both L-NA and 7-NI did not alter the number of superoxide generating cells. Y-axis denotes ratios of the superoxide or 3-NT–positive cells within neuron, astrocyte, and endothelial cell populations. Bars represent SD, *$P<0.05$ compared with control.

Colocalization of 3-Nitrotyrosine With Evans Blue Extravasation and MMP-9

Three (n=2) and 5 (n=2) hours after reperfusion, EB leakage was detectable around several microvessels, especially in the ischemic core (Figure 3E). Microvessels that became permeable to EB were almost all labeled with 3-NT (n=4,8) (Figure 3G). Since EB leakage had just started to emerge at these time points,[19] this colocalization suggests a causal relationship between peroxynitrite formation and microvascular injury. Supporting this view, 3-NT labeling was also colocalized with MMP-9 expression on the vessel wall 5 hours after reperfusion (n=2,4) (Figure 3H). MMP-9 expression was not at detectable levels 3 hours after reperfusion.

NOS Inhibition and Blood-Brain Barrier

A low dose of L-NA administered 15 minutes before reperfusion significantly reduced EB leakage 3 hours after reperfusion [0.33±0.06 versus 0.12±0.03 microgram/hemisphere in L-NA (n=6) and vehicle-treated (n=6) mice, respectively] (Figure 4). Contrary to the nonselective NOS inhibitor L-NA, 7-nitroindazole (7-NI) (in vivo selective inhibitor of nNOS) was not protective when given before reperfusion in our previous study and, hence, we proposed that NO of endothelial origin might be a major player in reperfusion-induced injury.[14] In the present study, taking advantage of double-labeling methods, we were able to investigate the inhibition of neuronal and endothelial peroxynitrite formation individually. L-NA (n=3,12) significantly decreased the number of 3-NT–labeled endothelia (17±4 versus 48±17%) and astrocytes (28±8 versus 51±10%) by 64 and 45%, respectively, compared with untreated mice (n=4,12) (Figure 2), but not the number of 3-NT–labeled neurons (72±17 versus 79±18%). On the other hand, 7-NI (n=3,12) given 30 minutes before reperfusion had no effect on 3-NT labeling in endothelial cells (45±9 versus 48±17%). Prereperfusion administration of both L-NA (n=3,9) and 7-NI (n=3,9) did not alter the number of Et+X-positive cells, suggesting that observed fluorescence reflected superoxide formation and was not altered by peroxynitrite. Details of distribution of both signals are given in Figure 2; however, it should be noted that counting of 3-NT or Et+X-labeled cells did not take into account the changes in signal intensity in individual cells, and hence, is only a semiquantitative measure.

Discussion

We detected superoxide and nitrotyrosine formation in neurons, astrocytes, and endothelial cells soon after reperfusion in intact brains. Double-labeling with several fluorescent probes gave us the opportunity to study cellular distribution of these molecules and interactions among them under in vivo conditions. Both signals were highly colocalized; since superoxide has a relatively low diffusibility, this finding suggests that NO, diffusing freely across membranes, reached to superoxide-generating foci and led to peroxynitrite formation.[4,5] Since there is no significant leukocyte infiltration and no inducible NOS activity in the mouse brain 3 hours after reperfusion,[20,21] we think that the major sources of NO were nNOS expressing neurons and the endothelium, whereas superoxide was generated within all 3 cell types.

High constitutive SOD activity may account for the relatively less numerous superoxide and peroxynitrite signals detected in the soma and processes of astrocytes.[22] Notably, however, astrocytic end-feet and endothelia were intensely

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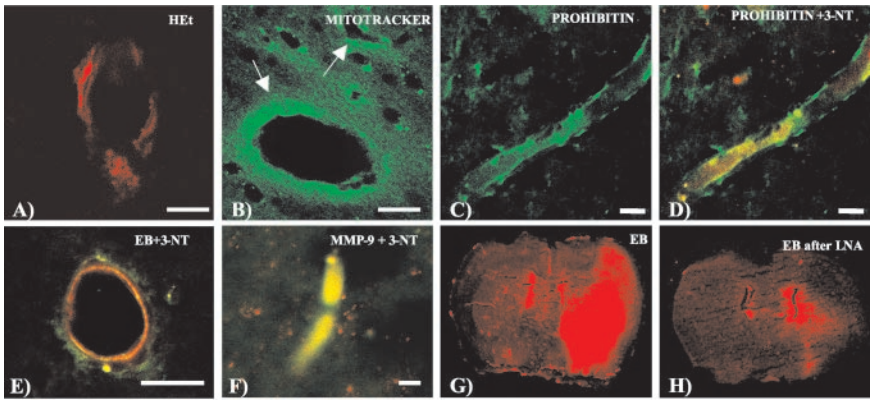

Figure 3. Reperfusion-induced superoxide and peroxynitrite formation causes vascular injury as illustrated by EB extravasation and MMP-9 expression. Three hours after recirculation, intense superoxide and 3-NT formation is seen in microvessels (A,D). These radical generating areas overlapped with high density of mitochondria (labeled with Mitotracker or antiprohibitin antibodies; B, C). 3-NT immunofluorescence (green) was colocalized (yellow) with EB leakage (red) and MMP-9 expression (red) (E, F), suggesting close association between peroxynitrite and microvascular injury. MMP-9 image was captured 5 hours postreperfusion. Supporting this, EB leakage (G) was reduced when peroxynitrite formation was inhibited just before reperfusion with L-NA (H). Scale bars=20 μm.

labeled for both anions. This area of intense radical formation overlapped with dense populations of mitochondria, which may be a significant source of superoxide on recirculation.[23] Endothelial enzymes can also contribute to superoxide generation.[24] eNOS has been reported to be a major source of NO during reperfusion, although it accounts for less than 10% of NO generation in normal mouse brain.[25] Therefore, NO produced by eNOS can diffuse and react with superoxide generated in end-feet and endothelia to form peroxynitrite, which may account for the intense 3-NT labeling in and around microvessels.[2,14,25]

The reaction between NO and superoxide was argued by some authors to promote protection by reducing superoxide toxicity, although this reaction occurs at the expense of generation of a stronger oxidant, peroxynitrite.[16] Moreover, peroxynitrite was reported to have beneficial effects in coronary circulation.[16,26] Therefore, we searched for evidence, whether or not peroxynitrite was indeed toxic to the cerebral microvasculature. We found that 3-NT signal was always colocalized with EB leakage or MMP-9 expression in microvessels after reperfusion, suggesting an association between peroxynitrite and vascular injury. Further supporting this idea, inhibition of NO synthesis by L-NA just before reperfusion decreased EB extravasation, consistent with suppression of MMP-9 expression with NOS inhibition in our previous report.[14] Additionally, by using double labeling methods, we have obtained direct evidence showing that the low-dose L-NA decreased 3-NT formation in endothelia and astrocytes, supporting our idea that partial inhibition of the

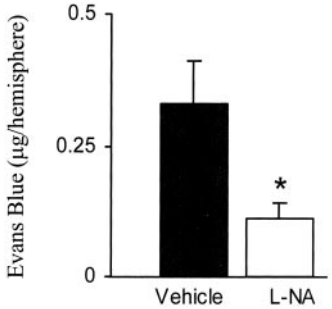

Figure 4. Partial inhibition of NOS activity with a low dose of L-NA (1 mg/kg, IP) 15 minutes before reperfusion significantly reduced EB leakage 3 hours after reperfusion *$P<0.05$, n=6 per group.

eNOS may contribute to preservation of BBB[14] by protecting vascular and end-feet integrity. These findings are in contrast to studies reporting solely a protective role for NO in coronary ischemia/reperfusion.[27] The discrepancy, in addition to differences in tissue biology (eg, urate generation in the heart may efficiently detoxify peroxynitrite),[28] may also be related to differences in vasculature (eg, tight endothelial junctions and astrocytic end-feet in the brain may be particularly vulnerable to oxidative/nitrative injury).[29–31] Moreover, loss of the selective permeability of BBB may jeopardize recovery of neural tissue after reperfusion.[32] Hence, the deleterious effects of NO during brain reperfusion may surpass its positive effects such as inhibition of platelet and leukocyte adhesion to endothelium.[15] Possibly because of this dual action of NO, high doses of NOS inhibitors increase infarct size, whereas low doses are protective.[33] Inhibition of peroxynitrite formation is also feasible by suppressing superoxide levels. In parallel to our results, Asahi et al found a decrease in reperfusion-induced vascular injury and reduction in hemorrhagic complications when a free radical scavenger was administered at reperfusion.[34] Similarly, SOD-overexpressing–transgenic mice were resistant to reperfusion injury, and studies with these mice suggested that the neurotoxic role of NO in ischemia/reperfusion depended on the reaction with superoxide to form peroxynitrite.[6,35]

Taken together, these findings suggest that superoxide, NO, and peroxynitrite formation in and around microvessels contribute to loss of selective BBB permeability and activation of MMPs. Supporting this idea, activation of MMP-9 via S-nitrosylation, and reduced MMP activation in mice overexpressing SOD have recently been reported.[36,37] MMP-mediated degradation of collagen and laminins in basal lamina disrupts the integrity of the vascular wall.[38,39] Oxidative/nitrative injury triggers recruitment and migration of white cells, which release enzymes that increase basal lamina degradation and vascular permeability.[20] These events eventually lead to parenchymal hemorrhage and vasogenic brain edema. Moreover, disruption of the BBB, and, hence, loss of regulation of selective supply of nutrients to the brain, may jeopardize recovery of already damaged cells. Therefore, coadministration of thrombolytic agents with free radical[34] or peroxynitrite scavengers,[40] or low doses of nonselective NO

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synthase inhibitors[14] may reduce reperfusion-induced injury and extend the therapeutic window for thrombolysis.

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