

Role of Endothelial Nitric Oxide Generation and Peroxynitrite Formation in Reperfusion Injury After Focal Cerebral Ischemia

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Background and Purpose—Reperfusion injury is one of the factors that unfavorably affects stroke outcome and shortens the window of opportunity for thrombolysis. Surges of nitric oxide (NO) and superoxide generation on reperfusion have been demonstrated. Concomitant generation of these radicals can lead to formation of the strong oxidant peroxynitrite during reperfusion.

Methods—We have examined the role of NO generation and peroxynitrite formation on reperfusion injury in a mouse model of middle cerebral artery occlusion (2 hours) and reperfusion (22 hours). The infarct volume was assessed by 2,3,5-triphenyl tetrazolium chloride staining; blood-brain barrier permeability was evaluated by Evans blue extravasation. Nitrotyrosine formation and matrix metalloproteinase-9 expression were detected by immunohistochemistry.

Results—Infarct volume was significantly decreased (47%) in animals treated with the nonselective nitric oxide synthase (NOS) inhibitor N^ω-nitro-L-arginine (L-NA) at reperfusion. The specific inhibitor of neuronal NOS, 7-nitroindazole (7-NI), given at reperfusion, showed no protection, although preischemic treatment with 7-NI decreased infarct volume by 40%. Interestingly, prereperfusion administration of both NOS inhibitors decreased tyrosine nitration (a marker of peroxynitrite toxicity) in the ischemic area. L-NA treatment also significantly reduced vascular damage, as indicated by decreased Evans blue extravasation and matrix metalloproteinase-9 expression.

Conclusions—These data support the hypothesis that in addition to the detrimental action of NO formed by neuronal NOS during ischemia, NO generation at reperfusion plays a significant role in reperfusion injury, possibly through peroxynitrite formation. Contrary to L-NA, failure of 7-NI to protect against reperfusion injury suggests that the source of NO is the cerebrovascular compartment. (*Stroke*. 2000;31:1974-1981.)

Key Words: cerebral ischemia, focal ■ matrix metalloproteinases ■ nitrates ■ nitric oxide ■ reperfusion injury

Restoration of blood flow in the territory of an occluded cerebral artery is feasible by thrombolytic therapy. However, application of thrombolysis is currently limited to the first 3 hours of ischemia because late administration of the fibrinolytic agent can lead to intracerebral hemorrhage and brain swelling.¹ These deleterious actions of delayed recirculation are attributed to reperfusion injury, a process that further damages ischemia-injured arterial wall as well as brain tissue.¹⁻³ Mechanisms of reperfusion injury are yet not well documented, but excess production of oxygen free radicals on restoration of oxygen supply is thought to play an important role.^{4,5} In support of this view, antioxidant agents have been shown to decrease reperfusion injury in animal models of transient ischemia in brain and other tissues.⁶

An increased production of nitric oxide (NO) in the brain has been demonstrated during ischemia.⁷⁻¹¹ Available evidence suggests that both neuronal and endothelial isoforms generate NO at the beginning of ischemia until the constitutive nitric oxide synthases (NOS) are inactivated.^{8,10-13} Al-

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though endothelial NO production is believed to positively affect the outcome of ischemia by improving ischemic blood flow, overproduction of NO in the brain parenchyma has been demonstrated to contribute to tissue damage.^{9,14,15} NO may be toxic by several mechanisms; however, formation of peroxynitrite after a reaction between NO and superoxide appears to be one of the major pathways leading to cell death.¹⁶⁻¹⁸ Constitutive NOS is reactivated on reperfusion, as indicated by resumption of NO generation.^{10,11} A concomitant surge in superoxide production may lead to peroxynitrite formation.^{5,16} Peroxynitrite is a powerful oxidant with a relatively long half-life and is more toxic than NO and superoxide individually.¹⁶ Hence, peroxynitrite has been proposed as one of the putative mediators of reperfusion injury.^{19,20}

We have tested this hypothesis in a mouse model of transient ischemia by inhibiting NO synthesis just before

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Physiological Parameters of the Experimental Groups

	Vehicle (n=10)	7-NI (rep) (n=6)	7-NI (isch) (n=4)	L-NA (rep) (n=7)	7-NI (isch)+L-NA (rep) (n=4)	Phenylephrine (n=5)
rCBF, %						
Ischemia	24±7	25±5	21±3	22±10	20±8	25±9
Reperfusion	92±20	75±18	93±38	93±31	104±33	86±32
MABP, mm Hg						
Ischemia	79±12	74±6	87±7	76±14	79±6	74±6
Reperfusion	76±10	75±7	86±9	92±16*	90±11*	90±18††
PaO ₂ , mm Hg	100±7	98±10	97±36	108±15	99±12	96±6
Paco ₂ , mm Hg	35±4	37±2	38±1	38±4	35±3	35±2
pH	7.38±0.05	7.33±0.09	7.34±0.07	7.39±0.02	7.37±0.07	7.35±0.03

In the column headings, abbreviations in parentheses denote time of administration of the agent: (rep) indicates reperfusion and (isch) ischemia. Values are mean±SD.

* $P < 0.05$.

††Not significantly different from the MABP values of L-NA-treated groups.

reperfusion after 2 hours of middle cerebral artery (MCA) occlusion. Our findings indicate that the nonselective NOS inhibitor *N*^ω-nitro-L-arginine (L-NA) but not a selective inhibitor of neuronal NOS (nNOS), 7-nitroindazole (7-NI), decreases infarct volume and Evans blue extravasation by ≈50%. In line with these data, L-NA diminished tyrosine nitration in cerebral vessels and brain tissue, possibly by inhibiting peroxynitrite formation.

Materials and Methods

Experimental Groups

Swiss Albino mice weighing 25 to 32 g were housed under diurnal lighting conditions (12 hours darkness and 12 hours light) and fasted overnight but allowed free access to water before the experiment. Animal housing, care, and application of experimental procedures were all done in accordance with institutional guidelines. Mice were anesthetized intraperitoneally with 400 mg/kg chloral hydrate. Body temperature was monitored by a rectal probe and maintained at 37.0±0.1°C by a homeothermic blanket control unit. A tubing placed toward the heart through the incision made on the common carotid artery for inserting the filament was used for continuous arterial blood pressure monitoring, for obtaining blood samples to determine pH, PCO₂, PO₂, and for heparinization (10 U just before induction of ischemia).

A total of 65 mice were studied. Thirty-six mice were subjected to 2 hours of proximal MCA occlusion and 22 hours of reperfusion and were studied in 6 groups: (1) 7 mice were injected intraperitoneally with L-NA 15 minutes before reperfusion; (2) 7-NI (50 mg/kg IP, dissolved in peanut oil) was given 30 minutes before ischemia (n=4) or (3) 30 minutes before reperfusion (n=6); (4) 4 mice were given 7-NI (50 mg/kg) 30 minutes before ischemia and L-NA (1 mg/kg) 15 minutes before reperfusion; (5) 5 mice were infused with phenylephrine (30 to 60 μg/h IV) for 30 minutes after reperfusion to increase arterial blood pressure to values comparable to those recorded in L-NA-treated groups; and (6) 10 mice received saline intraperitoneally 15 minutes before reperfusion.

Another set of 11 mice were subjected to 2 hours of ischemia and 5 hours of reperfusion for nitrotyrosine immunohistochemistry. For matrix metalloproteinase immunohistochemistry, 7 mice, and to evaluate Evans blue extravasation, 11 mice were subjected to 2 hours of ischemia and 22 hours of reperfusion.

MCA Occlusion

Proximal occlusion of the right MCA was performed with the use of a nylon filament, as described previously.⁹ Briefly, the right common carotid artery and external carotid artery were ligated by a 5-0 silk

suture after a midline incision. A nylon filament (8/0) was inserted into the common carotid artery through a small incision proximal to the bifurcation and advanced in the internal carotid artery up to the origin of MCA (10 mm from the bifurcation). The distal 3 mm of 8/0 filament was coated with silicon.

A flexible probe (PF-318 of PeriFlux PF 2B, Perimed) was placed over the skull (2 mm posterior, 6 mm lateral to the bregma), away from large pial vessels to monitor the regional cerebral blood flow (rCBF) by laser Doppler flowmetry. After obtaining a stable 10-minute epoch of preischemic rCBF, the MCA was occluded and rCBF was continuously monitored during ischemia (2 hours) and the first 20 to 40 minutes of reperfusion. Reperfusion was accomplished by pulling the filament back.

Evaluation of Ischemic Area by TTC Staining

The ischemic area was evaluated by TTC staining. Briefly, after an overdose of pentobarbital, mice were killed by decapitation after 2 hours of ischemia and 22 hours of reperfusion. The brains were quickly removed and placed in ice-cold saline for 5 minutes and then cut into 2-mm coronal slices. Sections were incubated in TTC-containing saline solution (2%, Sigma Chemical Co) for 20 minutes and in 10% formalin overnight. The infarction area, outlined in white, was measured by an image-analysis software (NIH Image 1.59) on the posterior surface of each section, and infarction volume was calculated by summing the infarct volume of sequential 2-mm-thick sections. Mice showing hippocampal involvement caused by circle of Willis anomalies²¹ were excluded from the study.

Neurological Evaluation

Twenty-two hours after recirculation, neurological deficits were assessed by an observer blinded to the identity of treatment and scored as described previously⁹: 0, no observable neurological deficits (normal); 1, failure to extend left forepaw on lifting the whole body by the tail (mild); 2, circling to the contralateral side (moderate); 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe).

Evans Blue Extravasation

Blood-brain barrier permeability was assessed by measuring Evans blue extravasation. Evans blue (Sigma, 0.1 mL of 4% solution) was injected into the tail vein at reperfusion.²² Mice were transcardially perfused with 100 mL of heparinized saline solution (10 IU/mL) 22 hours after reperfusion. Brains were removed and hemispheres were separated. Each hemisphere was well homogenized in 1 mL of 0.1 mol/L PBS and then centrifuged at 1000g for 15 minutes; 0.7 mL of 100% trichloroacetic acid was added into 0.7 mL of supernatant. The mixture was incubated at +4°C for 18 hours and then centrifuged at 1000g for 30 minutes. The amount of Evans blue in supernatant was

measured spectrophotometrically at 610-nm wavelength by comparison with readings obtained from standard solutions. Results were expressed as micrograms per hemisphere.

Immunohistochemistry

After 2 hours of ischemia and 5 hours of reperfusion, mice (4 treated with L-NA, 3 with 7-NI, and 4 with vehicle at reperfusion) were perfused transcardially with 4% paraformaldehyde solution. Brains were removed and kept in paraformaldehyde for 48 hours and were subsequently embedded in paraffin. After 4- μ m-thick slices were obtained, they were deparaffinized at 56°C overnight and hydrated in xylol and graded alcohol solutions. Sections were stained with anti-nitrotyrosine antibody (1/100 monoclonal, Upstate Biotechnology, Inc) by the avidin-biotin method.²³ To establish the specificity of antibody binding, 1 set of the tissue sections was incubated with antibody mixed with 15 mmol/L nitrotyrosine solution (dissolved in PBS and adjusted to pH 7.6) for 6 hours at room temperature.

After 2 hours of ischemia and 22 hours of reperfusion, 4- μ m-thick paraffin sections were prepared as above from 7 mice (3 treated with L-NA and 4 with vehicle) and were stained with anti-matrix metalloproteinase-9 (MMP-9, Calbiochem) antibody (1/50) by the avidin-biotin method, as described before.²⁴ Primary antibody was omitted to test the specificity of staining. Diaminobenzidine was used as chromogen and hematoxylin as counterstain for both antibodies.

Brain slices were evaluated for nitrotyrosine or MMP-9 staining by an observer blinded to the identity of treatment. Immunolabeled vessels in the ischemic area were counted manually over 100 contiguous complete microscopic fields (magnification $\times 400$) on sections taken 2.5 mm posterior of the frontal pole and passing through the anterior commissure.

Statistics

Mean values of arterial blood gases, pH, blood pressure, rCBF values, infarct volume, neurological examination scores, and number of vessels immunolabeled with nitrotyrosine antibody were compared by means of the Kruskal-Wallis test followed by the Mann-Whitney *U* test. The number of vessels immunolabeled with anti-MMP-9 antibody and amount of Evans blue extravasation between the vehicle and L-NA-treated groups were compared by means of the Mann-Whitney *U* test. Values of $P < 0.05$ were considered to be significant. Mean values in the text are given with their standard deviations.

Results

Arterial blood gases, pH, and pressure were not significantly different between the experimental groups, except that the groups treated with L-NA or phenylephrine had higher blood pressure values after drug administration (Table). rCBF values during MCA occlusion and reperfusion were not significantly different between groups.

Infarct Volume

L-NA treatment before reperfusion decreased the infarct volume by 47% (Figure 1A). Two-hour MCA occlusion followed by 22 hours of reperfusion caused an infarct of 75 ± 16 mm³ in vehicle-treated mice (n=10). Administration of 1 mg/kg of L-NA 15 minutes before reperfusion decreased the infarct volume to 40 ± 15 mm³ (n=7). 7-NI (50 mg/kg) showed no protection when administered 30 minutes before reperfusion (82 ± 22 mm³, n=6), although it reduced the infarct volume to 45 ± 4 mm³ when given 30 minutes before ischemia (n=4). Combining 7-NI (50 mg/kg) pretreatment with L-NA (1 mg/kg) administration before reperfusion (n=4) did not provide any further protection compared with L-NA treatment alone and reduced the infarct volume to

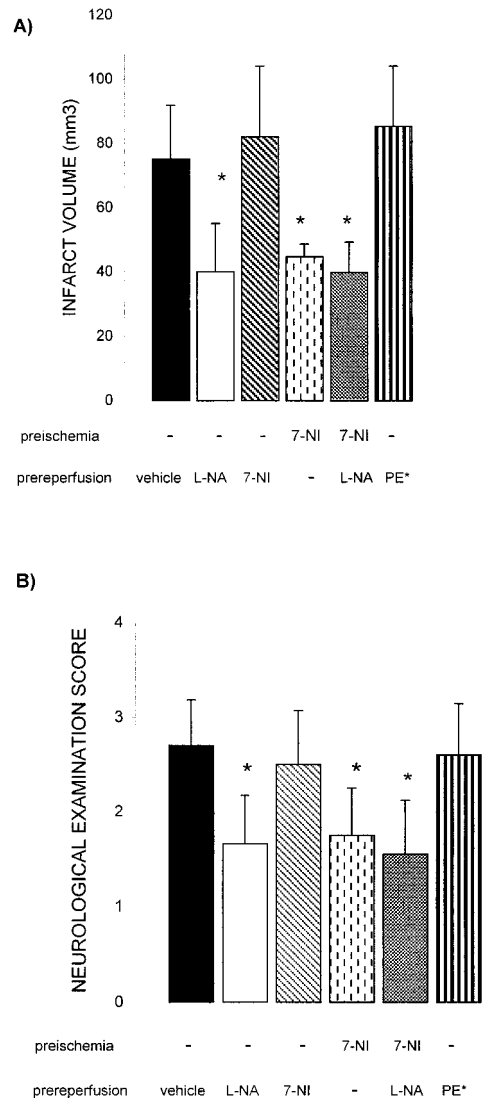


Figure 1. Infarct volume (A) and neurological examination scores (B) of control (filled columns) and treatment groups subjected to 2 hours of MCA occlusion and 22 hours of reperfusion. Values are mean \pm SD. Agents given and their time of administration are indicated below x-axis. * $P < 0.05$ compared with vehicle. PE*: Phenylephrine was infused for 30 minutes during reperfusion.

40 ± 9 mm³. A combined administration of 7-NI and L-NA 30 and 15 minutes (respectively) before reperfusion caused a high mortality rate during or shortly after surgery; therefore, the effect of this approach on reperfusion injury could not be tested. To test the effect of L-NA-induced modest blood pressure increase at reperfusion on infarct development, phenylephrine was infused intravenously starting at the beginning of reperfusion. Since mice started waking up from anesthesia after 30 minutes of reperfusion, the arterial blood pressure increased to values > 100 mm Hg in all groups irrespective of phenylephrine, L-NA, or vehicle administration; hence, phenylephrine infusion was given for only 30 minutes. The infusion rate (30 to 60 μ g/h) was adjusted so that phenylephrine increased the mean arterial pressure to values comparable to those recorded from L-NA-treated

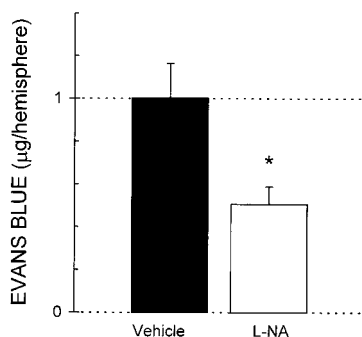


Figure 2. Amount of Evans blue extravasation in the ischemic hemisphere of control (filled column) and L-NA treatment group subjected to 2 hours of MCA occlusion and 22 hours of reperfusion. Values are mean \pm SD. * $P < 0.05$ compared with vehicle.

animals with a similar time course (Table). However, phenylephrine infusion showed no protection ($85 \pm 19 \text{ mm}^3$, $n=5$).

Neurological Evaluation

Mean neurological disability scores determined 22 hours after reperfusion paralleled the changes in infarct volume in all groups (Figure 1B). Treatment with L-NA before reperfusion and with 7-NI before ischemia significantly reduced disability scores compared with vehicle. The combined administration of 7-NI and L-NA led to a similar reduction in neurological dysfunction to L-NA treatment alone. Phenylephrine infusion provided no reduction in disability scores.

Evans Blue Extravasation

Evans blue extravasation 22 hours after reperfusion was $1.00 \pm 0.16 \mu\text{g}$ Evans blue per hemisphere in the vehicle-treated group ($n=5$). L-NA treatment before reperfusion led to a 50% reduction in extravasation ($0.50 \pm 0.08 \mu\text{g}$ per hemisphere, $n=6$, Figure 2).

Immunohistochemistry

Parenchyma and vessels in the ischemic territory were labeled with anti-nitrotyrosine antibody (Figure 3, A through F). This staining was considered to be specific because it was abolished by incubating the antibody with 3-nitrotyrosine or by omitting the antibody from the staining procedure, and brain sections from nNOS knockout mice subjected to permanent MCA occlusion for 6 hours (kindly provided by Dr M.A. Moskowitz of Massachusetts General Hospital, Boston) showed no immunolabeling. In preliminary experiments, we found that the intensity of ischemic changes and anti-nitrotyrosine staining were variable 1 hour after reperfusion. A dense staining was observed 5 hours after reperfusion, which declined by 22 hours. Therefore, we continued the study by comparing immunolabeling between groups 5 hours after reperfusion after 2 hours of ischemia.

At 5 hours, the intensity of ischemic changes were variable and patchy within the MCA territory. Nitrotyrosine staining closely paralleled the intensity of tissue damage, and intense labeling was prominent in the core region. Presumably glial cells (identified by light microscopic criteria) were negative, whereas neuronal cell bodies and neuropil were labeled (Figure 3, A through F). Immunolabeling of microvessels was more prominent than staining of neuropil and covered the

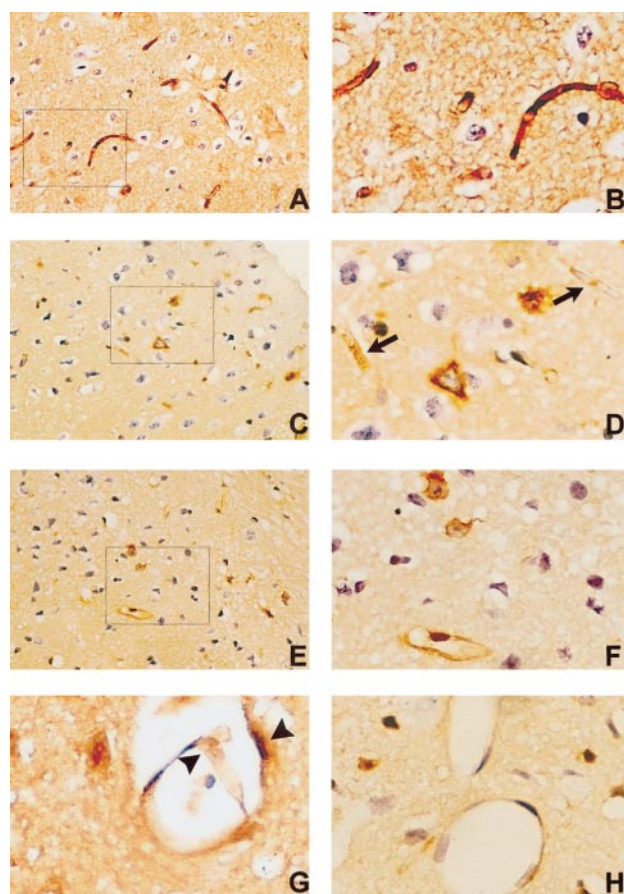


Figure 3. Reperfusion after 2 hours of MCA occlusion induced nitrotyrosine formation (A, B) and MMP-9 expression (G) in parenchyma and microvessels in the ischemic area. Anti-nitrotyrosine staining was performed 5 hours and anti-MMP-9 staining 22 hours after reperfusion. Note dense staining of the neuropil, some neurons, and microvessels with both antibodies. L-NA (C, D) and 7-NI (E, F) given before reperfusion decreased nitrotyrosine staining and L-NA decreased MMP-9 immunopositivity (H) compared with vehicle-treated mice (A, B and G). B, D, and F are higher-power ($\times 1000$) micrographs of the areas identified with a box in the lower-power ($\times 400$) micrographs in A, C, and E, respectively. Arrowheads in G denote the endothelium and vascular wall intensely expressing MMP-9. Note reduced MMP-9 expression and better preserved vascular structure in an L-NA-treated mouse (H). Arrows in D point to faintly stained and an immunonegative microvessel in an L-NA-treated mouse.

whole MCA area, being more intense in the core (Figure 3, A and B). Administration of both L-NA (Figure 3, C and D) and 7-NI (Figure 3, E and F) before reperfusion significantly but incompletely decreased nitrotyrosine immunolabeling of parenchyma and microvessels. Since quantification of parenchymal staining was less reliable compared with counting labeled microvessels, we compared the number immunolabeled microvessels in the ischemic hemisphere between groups. The number of immunopositive vessels was significantly less in L-NA-treated mice (644 ± 182 per section, $n=4$) compared with the vehicle-treated group (1920 ± 1040 per section, $n=4$). 7-NI treatment before reperfusion also significantly decreased the number of anti-nitrotyrosine-immunopositive vessels (691 ± 107 per section, $n=3$) (Figure 4A). We normalized the number of vessels for the area of ischemic region because the decrease in ischemic area after

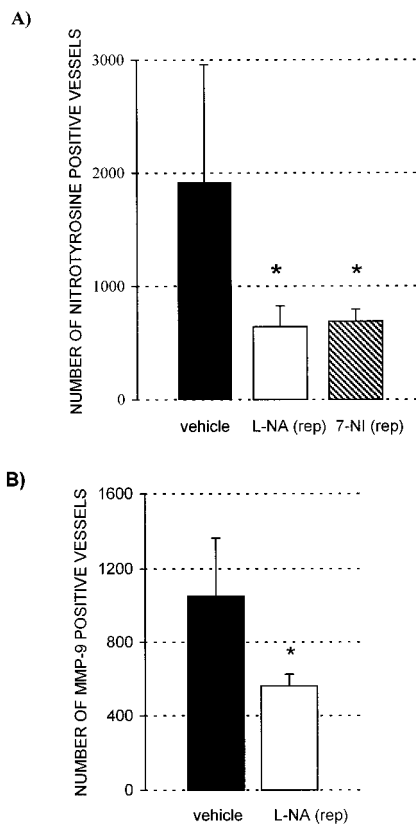


Figure 4. A, Number of immunolabeled vessels with anti-nitrotyrosine antibody 5 hours after reperfusion after 2 hours of ischemia. B, Number of vessels immunolabeled with anti-MMP-9 antibody after 22 hours of reperfusion after 2 hours of MCA occlusion. Immunopositive microvessels were counted on coronal sections passing through anterior commissure. * $P < 0.05$ compared with vehicle.

NOS inhibitors may contribute to the reduction in the number of immunolabeled vessels. Differences between groups were sustained after normalization: The number of vessels (in per mm² of ischemic area) was 155 ± 82 for vehicle ($n=4$), 71 ± 27 for L-NA ($n=4$), and 81 ± 30 for 7-NI treatment groups ($n=3$).

Twenty-two hours after reperfusion, anti-MMP-9 antibody positively labeled the parenchyma and vessels in the ischemic territory (Figure 3, G and H). The specificity of staining was tested by omitting the antibody from the staining procedure. The number of immunolabeled vessels was decreased in the L-NA-treated group (561 ± 64 , $n=3$) compared with the saline-treated group (1050 ± 314 , $n=4$) (Figure 4B).

Discussion

Nowicki and his colleagues²⁵ were the first to report that low doses of L-NA were neuroprotective in a mouse model of permanent MCA occlusion. Recent evidence indicates that low doses of nonselective NOS inhibitors also have a wide therapeutic window in transient MCA occlusion models.^{26–29} Our findings are in line with these observations and show that L-NA significantly reduced ischemic brain damage when given just before reperfusion after 2 hours of ischemia, whereas 7-NI did not have any anti-ischemic action at this time point, although its preischemic administration was

equally protective. Similarly, Escott et al³⁰ reported that 7-NI did not provide protection when administered 30 minutes before reperfusion in rats subjected to 2 hours of transient MCA occlusion, whereas 7-NI given at the beginning of ischemia decreased infarct volume by 38.5%. The detrimental role of NO generated by nNOS in ischemic injury is well established^{9,15}; however, selective inhibition of nNOS appears to have a short therapeutic window,^{14,30,31} contrary to nonselective NOS inhibitors. Failure of 7-NI given before reperfusion, while L-NA is effective, suggests that the source of NO that contributes to reperfusion injury is essentially the vascular endothelium. Inducible NOS (iNOS) is unlikely to be a major source of NO because 2 hours after ischemia, there is no significant iNOS expression either in the parenchyma or vascular wall.^{12,32,33} However, leukocytes adhering to endothelium may also be another source of NO and superoxide during reperfusion.^{2,5,34,35}

One possibility is that 7-NI may have failed because of an insufficient inhibition of neuronal NO synthesis. 7-NI (25 to 60 mg/kg IP) has been shown to inhibit brain NOS activity rapidly (reaching a maximum in 15 to 30 minutes) but incompletely (47% to 65%) in the mouse and rat.^{14,36,37} However, intraperitoneal administration of 1 mg/kg L-NA also inhibits brain NOS activity partially (maximum 74%).³⁸ Hence, the major difference between the 2 inhibitors at the time of reperfusion appears to be the inhibition endothelial NOS (eNOS) by L-NA but not 7-NI, as also indicated by changes in blood pressure (see References 14, 36, and 37).

We have also excluded the possibility that the L-NA-induced blood pressure increase may have provided a better reperfusion and a more favorable outcome by showing that a comparable increase in blood pressure obtained by phenylephrine infusion had no neuroprotective effect. Although statistically not significantly different than rCBF values in other groups, slightly lower rCBF values during reperfusion in the 7-NI–prereperfusion treatment group may be thought to have masked any beneficial effect of 7-NI. However, the phenylephrine study indicates that moderate hemodynamic changes during reperfusion after 2 hours of MCA occlusion are not so critical as they are during ischemia. In fact, the group given combined 7-NI and L-NA treatments had higher blood pressure and rCBF values during reperfusion, yet displayed no additional benefit that could be attributed to 7-NI. Taken together, we can conclude that the protective action of L-NA is unlikely to be due to pharmacokinetic or hemodynamic factors but rather points to the importance of inhibition of vascular sources of NO. Recently, Wei et al,³⁹ by using nNOS and eNOS knockout mice, showed that eNOS accounted for half of the NO surge seen during reperfusion after global ischemia. If the eNOS is comparably overactive during reperfusion after MCA occlusion, L-NA will clearly provide a superior protection compared with 7-NI by blocking eNOS as well.

Several laboratories that used various chemical detection methods have demonstrated that brain NO level increases at the beginning of focal ischemia.^{7–11,15} This increase is not sustained during the course of ischemia, possibly because of inactivation of constitutive NOS.^{8,12,13} However, NO synthesis resumes on reperfusion.^{10,11} Kumura et al⁴⁰ detected a

surge in hemoglobin-NO, nitrite, and nitrate levels in jugular blood during reperfusion after 2-hour MCA occlusion. An excessive superoxide production was also reported after reperfusion.⁵ Administration of superoxide dismutase (SOD) further increased the hemoglobin-NO signal at reperfusion in the study by Kumura and colleagues,⁴⁰ indicating that some of the NO produced was converted to peroxynitrite by reacting with superoxide during reperfusion. In line with these findings, a second surge of nitrotyrosine formation was detected in the penumbral cortex after reperfusion.²⁰ Although current methods have shortcomings to unequivocally establish the compartment that peroxynitrite formation takes place, the study by Kumura and colleagues⁴⁰ indicates that the intimate colocalization between superoxide and NO generation required for formation of peroxynitrite is realized in the vascular compartment.

Supporting the above view, we have seen an intense nitrotyrosine immunostaining in vascular walls, including the endothelium within the ischemic area. Neurons and neuropil were also densely stained, and 7-NI significantly decreased vascular as well as parenchymal staining, suggesting that nNOS in neurons and perivascular nerves contributed to peroxynitrite formation and nitration of tyrosine residues. It is surprising that 7-NI was not protective against reperfusion injury, although it decreased nitrotyrosine (hence peroxynitrite) formation to an extent comparable to L-NA administration. Although these findings suggest a nonperoxynitrite-mediated, yet unidentified NO-related mechanism, the possibility that staining of critical targets may have been masked by labeling of free and other protein-bound nitrotyrosine should be considered. It is likely that peroxynitrite generation within the vascular endothelium may specifically target some critical molecules to disrupt vascular integrity and increase microvascular permeability.^{41,42} Compartmentalization of these target molecules within the vascular wall or localization of detoxification mechanisms (eg, SOD) or local tissue factors (eg, pH) may hinder nNOS-generated peroxynitrite to reach these vascular targets, although peroxynitrite has the ability to diffuse and damage distant molecules.^{16,43} Absence of immunoreactivity in some cells (presumably glia) next to the intensely stained neurons and microvessels points to the importance of local cellular factors and rate of nitrotyrosine removal in determining the outcome of peroxynitrite-induced injury. Astrocytes have high SOD levels, whereas neurons as well as vascular cells have been reported to express NADPH oxidase, which may be a significant source of oxidative stress.^{4,44} Removal of nitrotyrosine appears to be particularly important because more peroxynitrite is generated in the penumbra, as detected by HPLC,^{16,20,43} but nitration is possibly quickly restored and escapes detection by immunohistochemistry, whereas nitrotyrosine remains bound to proteins in the core. Complex and pH-dependent chemistry between peroxynitrite, NO, and nitrite may also contribute to this discrepancy; however, these reactions are yet not well characterized in vivo to correctly anticipate the net outcome.⁴³

It is tempting to speculate that eNOS may generate both NO and superoxide as the result of ischemia-induced L-arginine or tetrahydrobiopterin depletion,^{15,45,46} and this

may provide the optimum colocalization for a high throughput peroxynitrite production within the endothelium that (perhaps together with an additional source coming from the leukocytes adhered to endothelium) will especially damage vascular targets. In support of this view, L-NA not only reduced the infarct volume but also decreased blood-brain barrier leakage as detected by Evans blue extravasation. A reduced MMP-9 expression also indicates better preserved vascular integrity in L-NA-treated animals. The vascular basement membrane plays a critical role in maintaining integrity of the blood-brain barrier by providing structural support to the endothelial wall. Recently, an increase in activity of matrix metalloproteinases (a group of enzymes that degrade proteins of the extracellular matrix) and a decrease in extracellular matrix molecules such as type IV collagen, laminin, and fibronectin after ischemia/reperfusion have been demonstrated.^{3,24} In these studies, MMP-9 was detected in the ischemic tissue within 2 to 6 hours after ischemia and was markedly expressed at 24 hours.^{24,47}

Apart from its toxicity by leading to peroxynitrite formation, NO synthesized in the endothelium may positively affect the outcome by decreasing platelet and white blood cell clogging during reperfusion.³⁴ L-NA has been reported to have a U-shaped effect when administered during reperfusion.²⁹ The unfavorable action seen with high doses of L-NA may be due to profound inhibition of eNOS, promoting cellular adhesion. In fact, administration of NO donors during reperfusion was reported to decrease tissue damage after peripheral arterial occlusion.³⁵ The dose we used caused a modest increase in blood pressure, indicating that L-NA only partially inhibited eNOS. The partial inhibition of eNOS therefore may provide the optimum benefit by inhibiting peroxynitrite formation (or yet an unidentified NO-related mechanism) without significantly increasing intravascular clogging.

In conclusion, these data support the hypothesis that NO generation at reperfusion plays an important role in reperfusion injury, possibly by peroxynitrite formation. Contrary to L-NA, failure of 7-NI to protect against reperfusion injury suggests that the source of NO is the vascular compartment.

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References

1. Brott T, Broderick JP, Kothari R. Thrombolysis for acute ischemic stroke. In: Welch KMA, Caplan LR, Reis DJ, Siesjo BK, Weir Bryce, eds. *Primer on Cerebrovascular Disease*. San Diego, Calif: Academic Press; 1997:728–731.
2. Aronowski J, Strong R, Grotta JC. Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. *J Cereb Blood Flow Metab*. 1997;17:1048–1056.
3. Hamann GF, Okada Y, Fitridge Y, del Zoppo GJ. Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. *Stroke*. 1995;26:2120–2126.
4. Chan PH. Role of oxidants in ischemic brain damage. *Stroke*. 1996;27:1124–1129.
5. Peters O, Back T, Lindauer U, Busch C, Megow D, Dreier J, Dirnagl U. Increased formation of reactive oxygen species after permanent and

- reversible middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab.* 1998;18:196–205.
6. Hall ED. Antioxidant pharmacotherapy. In: Ginsberg M, Bogousslavsky J, eds. *Cerebrovascular Disease: Pathophysiology, Diagnosis and Management*. Boston, Mass: Blackwell Science; 1997:710–720.
 7. Tomimaga T, Sato S, Ohnishi T, Ohnishi ST. Potentiation of nitric oxide formation following bilateral carotid occlusion and focal cerebral ischemia in the rat: in vivo detection of the nitric oxide radical by electron paramagnetic resonance spin trapping. *Brain Res.* 1993;614:342–346.
 8. Kader A, Frazzini VI, Solomon RA, Trifiletti RR. Nitric oxide production during focal cerebral ischemia in rats. *Stroke.* 1993;24:1709–1716.
 9. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science.* 1994;265:1883–1885.
 10. Zhang ZG, Chopp M, Bailey F, Malinski T. Nitric oxide changes in the rat brain after transient middle cerebral artery occlusion. *J Neurol Sci.* 1995;128:22–27.
 11. Ohta K, Graf R, Rosner G, Heiss W-D. Profiles of cortical tissue depolarization in cat focal cerebral ischemia in relation to calcium ion homeostasis and nitric oxide production. *J Cereb Blood Flow Metab.* 1997;17:1170–1181.
 12. Grandati M, Verrecchia C, Revaud ML, Allix M, Boulu RG, Plotkine M. Calcium-independent NO synthase activity and nitrites/nitrates production in transient focal cerebral ischaemia in mice. *Br J Pharmacol.* 1997;122:625–630.
 13. Sporer B, Martens KH, Koedel U, Haberl RL. L-arginine-induced regional cerebral blood flow increase is abolished after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab.* 1997;17:1074–1080.
 14. Yoshida T, Limmroth V, Irikura K, Moskowitz MA. The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J Cereb Blood Flow Metab.* 1994;14:924–929.
 15. Dalkara T, Endres M, Moskowitz MA. Mechanisms of NO neurotoxicity. In: Mize RR, Dawson TM, Dawson VL, Friedlander MJ, eds. *Prog Brain Res.* 1998;118:231–239.
 16. Beckman JS. Interactions of oxidants, nitric oxide, and antioxidant defenses in cerebral ischemia and injury. In: Ginsberg MD, Bogousslavsky J, eds. *Cerebrovascular Disease*. Boston, Mass: Blackwell Science; 1998:455–470.
 17. Eliasson MJL, Huang Z, Ferrante RJ, Sasamata M, Molliver ME, Snyder SH, Moskowitz MA. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J Neurosci.* 1999;19:5910–5918.
 18. Forman LJ, Liu P, Nagele RG, Yin K, Wong PY. Augmentation of nitric oxide, superoxide, and peroxynitrite production during cerebral ischemia and reperfusion in the rat. *Neurochem Res.* 1998;23:141–148.
 19. Takizawa S, Fukuyama N, Hirabayashi H, Nakazawa H, Shinohara Y. Dynamics of nitrotyrosine formation and decay in rat brain during focal cerebral ischemia-reperfusion. *J Cereb Blood Flow Metab.* 1999;19:667–672.
 20. Fukuyama N, Takizawa S, Ishida H, Hoshiai K, Shinohara Y, Nakazawa H. Peroxynitrite formation in focal cerebral ischemia-reperfusion in rats occurs predominantly in peri-infarct region. *J Cereb Blood Flow Metab.* 1998;18:123–129.
 21. Özdemir YG, Bolay H, Erdem E, Dalkara T. Occlusion of the MCA by an intraluminal filament may cause disturbances in the hippocampal blood flow due to anomalies of circle of Willis and filament thickness. *Brain Res.* 1999;822:260–264.
 22. Kondo T, Reaume AG, Huang T-T, Carlson E, Murakami K, Chen SF, Hoffman EK, Scott RW, Epstein CJ, Chan PH. Reduction of CuZn-Superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia. *J Neurosci.* 1997;17:4180–4189.
 23. Beckman JS, Ye YZ, Anderson P, Chen J, Accavitti MA, Tarpey MM, White CR. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe-Seyler.* 1994;375:81–88.
 24. Gasche Y, Fujimura M, Morita-Fujimura Y, Copin J-P, Kawase M, Massengale J, Chan PH. Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood-brain barrier dysfunction. *J Cereb Blood Flow Metab.* 1999;19:1020–1028.
 25. Nowicki JP, Duval D, Poignet H, Scatton B. Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. *Eur J Pharmacol.* 1991;204:239–240.
 26. Ashwal S, Cole DJ, Osborne TN, Pearce WJ. Dual effects of L-NAME during transient focal cerebral ischemia in spontaneously hypertensive rats. *Am J Physiol.* 1994;267:H276–H284.
 27. Margail I, Allix M, Boulu RG, Plotkine M. Dose- and time-dependence of L-NAME neuroprotection in transient focal cerebral ischaemia in rats. *Br J Pharmacol.* 1997;120:160–163.
 28. Matsui T, Nagafuji T, Mori T, Asano T. N-omega-nitro-L-arginine attenuates early ischemic neuronal damage of prolonged focal cerebral ischemia and recirculation in rats. *Neurol Res.* 1997;19:192–203.
 29. Spinnewyn B, Cornet S, Auguet M, Chabrier P-E. Synergistic protective effects of antioxidant and nitric oxide synthase inhibitor in transient focal ischemia. *J Cereb Blood Flow Metab.* 1999;19:139–143.
 30. Escott KJ, Beech JS, Haga KK, Williams SC, Meldrum BS, Bath PM. Cerebroprotective effect of the nitric oxide synthase inhibitors, 1-(2-trifluoromethylphenyl)imidazole and 7-nitro indazole, after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab.* 1998;18:281–287.
 31. Zhang ZG, Reif D, MacDonald J, Tang WX, Kamp DK, Gentile RJ, Shakespeare WC, Murray RJ, Chopp M. ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab.* 1996;16:599–604.
 32. Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab.* 1995;15:378–384.
 33. Yoshida T, Waeber C, Huang Z, Moskowitz MA. Induction of nitric oxide synthase activity in rodent brain following middle cerebral artery occlusion. *Neurosci Lett.* 1995;194:214–218.
 34. Niu XF, Smith CW, Kubes P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ Res.* 1994;74:1133–1140.
 35. Johnson D, Freischlag JA, Lesniak R, Kelly H, Mudaliar JH, Cambria RA, Seabrook MD, Towne JB. Endothelial damage due to ischemia and reperfusion is prevented with SIN-1. *Cardiovasc Surg.* 1998;6:527–532.
 36. Moore PK, Wallace P, Gaffen Z, Hart SL, Babbedge RC. Characterization of the novel nitric oxide synthase inhibitor 7-nitro indazole and related indazoles: antinociceptive and cardiovascular effects. *Br J Pharmacol.* 1993;110:219–224.
 37. Stagliano NE, Dietrich WD, Prado R, Green EJ, Busto R. The role of nitric oxide in the pathophysiology of thromboembolic stroke in the rat. *Brain Res.* 1997;6:759:32–40.
 38. Carreau A, Duval D, Poignet H, Scatton B, Vige X, Nowicki JP. Neuroprotective efficacy of N omega-nitro-L-arginine after focal cerebral ischemia in the mouse and inhibition of cortical nitric oxide synthase. *Eur J Pharmacol.* 1994;256:241–249.
 39. Wei G, Dawson VL, Zweier JL. Role of neuronal and endothelial nitric oxide synthase in nitric oxide generation in the brain following cerebral ischemia. *Biochim Biophys Acta.* 1999;1455:23–34.
 40. Kumura E, Yoshimine T, Iwatsuki K-I, Yamanaka K, Tanaka S, Hayakawa T, Shiga T, Kosaka H. Generation of nitric oxide and superoxide during reperfusion after focal cerebral ischemia in rats. *Am J Physiol.* 1996;270:C748–C752.
 41. Greenacre S, Ridger V, Wilsoncroft P, Brain SD. Peroxynitrite: a mediator of increased microvascular permeability. *Clin Exp Pharmacol Physiol.* 1997;24:880–882.
 42. Gobbel GT, Chan TYY, Chan PH. Nitric oxide and superoxide-mediated toxicity in cerebral endothelial cells. *J Pharmacol Exp Ther.* 1997;282:1600–1607.
 43. Crow JP, Spruell C, Chen J, Gunn C, Ischiropoulos H, Tsai M, Smith CD, Radi R, Koppenol WH, Beckman JS. On the pH-dependent yield of hydroxyl radical products from peroxynitrite. *Free Radic Biol Med.* 1994;16:331–338.
 44. Tammariello SP, Quinn MT, Estus S. NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. *J Neurosci.* 2000;20:RC53:1–5.
 45. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc Natl Acad Sci U S A.* 1996;93:6770–6774.
 46. Milstein S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun.* 1999;263:681–684.
 47. Mun-Bryce S, Rosenberg GA. Matrix metalloproteinases in cerebrovascular diseases. *J Cereb Blood Flow Metab.* 1998;18:1163–1172.

Editorial Comment

For some time, evidence has been accumulating that the cerebrovasculature is a major site of oxidative damage in stroke.¹ I have long been puzzled how injection of polyethylene glycol-conjugated SOD into the circulation could reduce infarction after focal ischemia.² This large protein complex is impermeable to the blood-brain barrier, making the cerebral microvessels a likely site for its protective effects. In the present study by Gürsoy-Özdemir et al, a moderately low dosage of nitroarginine, a relatively nonselective inhibitor of NO synthesis, was found to greatly reduce infarct volume in mice subjected to focal ischemia. In contrast, larger dosages of 7-NI, which is putatively more selective for nNOS in vivo,³ was not protective. The authors suggest that partial inhibition of the eNOS in the cerebral circulation may be protective against reperfusion injury.

The pharmacological evidence using 7-NI must be carefully interpreted, because 7-NI is a potent inhibitor of eNOS in vitro. The apparent selectivity for nNOS in vivo may result from the metabolism of the purine-based 7-NI by xanthine dehydrogenase, which is enriched in brain endothelium.⁴ Alternatively, export of 7-NI by purine transporters may limit the intracellular accumulation in endothelium.⁴ However, a role of NO-dependent injury to brain microvessels was further supported in the present study by observation of increased nitration of tyrosine in the majority of cerebral microvessels in the ischemic territory. This tyrosine nitration was found to increase several hours after reperfusion had been initiated. Nitric oxide itself does not nitrate proteins but can form the much stronger oxidant peroxynitrite by a diffusion-limited reaction with superoxide. Although other reactive nitrogen species can contribute to a fraction of nitrotyrosine formation in vivo, peroxynitrite remains the most efficient source of tyrosine nitration in proteins in biological systems.^{5,6}

Immunoprecipitation of proteins with nitrotyrosine antibodies has already identified several targets that could be important in the disruption of cerebrovascular function. Manganese SOD is the major enzymatic scavenger of superoxide in mitochondria and is readily nitrated and inactivated by peroxynitrite in vivo.^{7,8} The sarcoplasmic calcium ATPase found in vascular smooth muscle is inactivated by nitration and has major effects in the control of muscle contractility. Prostacyclin synthase is rapidly inactivated by submicromolar concentrations of peroxynitrite in endothelium.⁹⁻¹¹ After exposure to peroxynitrite, prostacyclin synthase produces thromboxane-like intermediates that promote vasoconstriction and platelet aggregation.^{12,13} Furthermore, peroxynitrite appears to be the physiological peroxide necessary to activate cyclooxygenase.¹⁴ Consequently, a sustained production of peroxynitrite in the vasculature may strongly promote thromboxane synthesis, disrupt calcium handling to affect contractile strength, and injure mitochondria necessary to

maintain the integrity of the blood-brain barrier. These actions would likely promote cerebral injury after stroke.

The narrow time window for successful TPA therapy has greatly limited its success in treating stroke. However, we have only just begun to appreciate the complex secondary effects of oxidative damage induced in the brain microcirculation after cerebral ischemia. The brain endothelium offers the most readily accessible target for therapeutic intervention in the treatment of stroke. Based on the time course of tyrosine nitration reported in the present study, agents to reduce oxidative damage in the microvasculature could potentially extend the therapeutic window in treating stroke by many hours.

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References

1. Kontos HA. George E. Brown memorial lecture: oxygen radicals in cerebral vascular injury. *Circ Res.* 1985;57:508-516.
2. Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am J Physiol.* 1989;256:H589-H93.
3. Moore PK, Babbedge RC, Wallace P, Gaffen ZA, Hart SL. 7-Nitro indazole, an inhibitor of nitric oxide synthase, exhibits anti-nociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol.* 1993;108:296-297.
4. Betz LA. Identification for hypoxanthine transport and xanthine oxidase activity in brain capillaries. *J Neurochem.* 1985;44:574-579.
5. Beckman JS. Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol.* 1996;9:836-844.
6. Sampson JB, Ye YZ, Rosen H, Beckman JS. Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch Biochem Biophys.* 1998;356:207-213.
7. MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci U S A.* 1996;93:11853-11858.
8. MacMillan-Crow LA, Crow JP, Thompson JA. Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry.* 1998;37:1613-1622.
9. Zou M-H, Ullrich V. Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. *FEBS Lett.* 1996;382:101-104.
10. Zou M, Martin C, Ullrich V. Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. *Biol Chem Hoppe Seyler.* 1997;378:707-713.
11. Zou MH, Klein T, Pasquet JP, Ullrich V. Interleukin 1beta decreases prostacyclin synthase activity in rat mesangial cells via endogenous peroxynitrite formation. *Biochem J.* 1998;336:507-512.
12. Viner RI, Ferrington DA, Williams TD, Bigelow DJ, Schöneich C. Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca²⁺-ATPase in skeletal muscle. *Biochem J.* 1999;340:657-669.
13. Viner RI, Ferrington DA, Hühmer AFR, Bigelow DJ, Schöneich C. Accumulation of nitrotyrosine on the SERCA2a isoform of SR Ca-ATPase of rat skeletal muscle during aging: a peroxynitrite-mediated process? *FEBS Lett.* 1996;379:286-290.
14. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc Natl Acad Sci U S A.* 1996;93:15069-15074.