

Peroxynitrite Diminishes Myogenic Activity and Is Associated With Decreased Vascular Smooth Muscle F-Actin in Rat Posterior Cerebral Arteries

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Background and Purpose—This study investigated the effect of peroxynitrite (ONOO^-) on pressure-induced myogenic activity and vascular smooth muscle (VSM) actin of isolated posterior cerebral arteries (PCAs).

Methods—Histochemical staining of nitrotyrosine (NT) was used to demonstrate the presence of ONOO^- in the cerebrovasculature after 1 hour of middle cerebral artery occlusion with 30 minutes of reperfusion. To determine the effect of ONOO^- on pressure-induced myogenic activity, third-order PCAs from nonischemic animals were isolated and mounted in an arteriograph chamber. Diameter in response to changes in pressure was determined in the absence and presence of ONOO^- (10^{-8} to 10^{-4} mol/L). Filamentous actin (F-actin) and globular actin (G-actin) were quantified using confocal microscopy in PCAs with and without exposure to ONOO^- .

Results—NT staining of vascular cells was greater in ischemic brain versus sham animals ($56 \pm 3\%$ versus $35 \pm 3\%$; $P < 0.01$). Addition of low concentrations of ONOO^- ($\leq 10^{-6}$ mol/L) to isolated PCAs caused constriction from $129 \pm 16 \mu\text{m}$ to $115 \pm 15 \mu\text{m}$ ($P < 0.01$), whereas concentrations $> 10^{-6}$ mol/L caused dilation of spontaneous tone and loss of myogenic activity in the physiological range of 50 to 125 mm Hg, increasing diameter from 130 ± 6 to $201 \pm 5 \mu\text{m}$ at 75 mm Hg ($P < 0.01$). In addition, the diminished myogenic activity was associated with a 4.5-fold decrease in F-actin content of VSM and a 27% increase in G-actin content ($P < 0.01$).

Conclusions—This study demonstrates that ONOO^- affects the myogenic activity of cerebral arteries and causes F-actin depolymerization in VSM, a consequence that could promote vascular damage during reperfusion injury and further brain injury. (*Stroke*. 2006;37:894-899.)

Key Words: actins ■ cerebral arteries ■ peroxynitrous acid ■ reperfusion injury

Our previous studies showed that ischemia and reperfusion (I/R) has a significant effect on the cerebral vasculature, including decreased myogenic tone and reactivity that could promote further brain injury and disrupt cerebral blood flow.¹⁻³ Our group also showed that loss of myogenic activity after I/R was associated with a decrease in filamentous actin (F-actin) in vascular smooth muscle (VSM).⁴ Because the polymerization state of the actin cytoskeleton is a significant contributor to pressure-induced myogenic activity,^{5,6} it is possible that the loss of tone during I/R is attributable to an effect on the actin cytoskeleton of VSM.

Peroxynitrite (ONOO^-) is a reactive oxygen species produced by the interaction of NO and superoxide (O_2^-) during cerebral I/R.⁷⁻⁹ ONOO^- acts as an oxidant, is more stable than NO or O_2^- , and can readily diffuse across phospholipid membranes.^{7,8,10} It has also been shown to nitrosylate the actin cytoskeleton and inhibit polymerization in leukocytes,¹¹ suggesting ONOO^- can affect the contractile apparatus. In fact, ONOO^- has been shown to affect the contractile state of

large cerebral arteries.¹² However, its effect on pressure-induced myogenic activity and VSM actin in small cerebral arteries is largely unknown. We therefore investigated the effect of ONOO^- on spontaneous myogenic tone and reactivity of isolated and pressurized posterior cerebral arteries (PCAs) and quantified F-actin and globular actin (G-actin) in VSM using confocal laser microscopy.

Materials and Methods

Animals

Male Wistar rats (350 to 450 g; n=22; Harlan, Indianapolis, Ind) were used for all experiments. Animals were housed in the institutional animal care facility and food and water supplied ad libitum. All procedures were approved by the institutional animal care and use committee.

Middle Cerebral Artery Occlusion Model

To determine the presence of ONOO^- during I/R in the cerebral vasculature, nitrotyrosine (NT) was used as a marker for ONOO^- .¹³ I/R was produced by temporary filament occlusion of the right

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middle cerebral artery (MCA), as described previously.^{1-4,14} The MCA was occluded for 1 hour followed by 30 minutes of reperfusion (n=3). The same surgical procedure to carotid exposure, without interruption of blood flow, was used for shams (n=3).

NT Staining

After MCA occlusion (MCAO) and while still under anesthesia, animals were fixed by cardiac perfusion with paraformaldehyde. Fixed brains were paraffin embedded and stained for NT with anti-NT (Upstate; 05-233) and EnV+Dual Link mouse anti-rabbit horseradish peroxidase (HRP; DakoCytomation, Inc.) and hematoxylin using standard staining techniques. Brain sections were viewed on an Olympus AX70 microscope, images captured with an Optronics MagnaFire digital camera, and then analyzed for HRP staining using Adobe Photoshop. Ipsilateral and contralateral sides of brain sections were analyzed for the ratio of NT-positive (brown-HRP) to negative (blue-hematoxylin) vascular cells by counting 12 vessels from each group and side within the MCA territory. Counting was done blinded to the experimental group. Only vascular cells were counted, and distinction between smooth muscle and endothelial layers was not made. Vascular cells were identified through cell organization and morphology using light microscopy. Positive NT cells were identified by characteristic brown HRP staining, whereas NT-negative cells were blue, which were clearly distinct (Figure 1A). Counting was repeated and the results averaged.

Preparation of Cerebral Arteries and Pressurized Arteriograph System

Nonischemic animals were used for all reactivity studies using an arteriograph chamber according to methods established previously.^{1-3,15} PCAs were used for the in vitro investigations because they develop consistent and significant myogenic tone and reactivity to pressure. The arteriograph chamber (Living Systems Instrumentation) was connected to an 80-mL reservoir and heat exchanger that continuously recirculated Hepes physiological saline solution (Hepes PSS) at a constant temperature of $37 \pm 0.03^\circ\text{C}$ and allowed for the addition

of drugs (eg, ONOO^-). The distal cannula was closed off so there was no luminal flow.

F-Actin and G-Actin Staining and Quantification

PCAs pressurized at 75 mm Hg and fixed in the absence or presence of 10^{-4} mol/L ONOO^- were stained for F-actin (n=8) using phalloidin-Oregon Green 488 or G-actin (n=5) with DNase I-Alexa-488 (Molecular Probes, Inc.) using established techniques.^{4,6} Arteries were viewed with a Zeiss LSM 510 laser scanning confocal microscope at $40\times$. Optimal gain for all groups was based on PCAs with spontaneous tone at 75 mm Hg. Then $40\times$ images focused on the VSM at a depth of $1.5 \mu\text{m}$ from the abluminal surface and centered on the artery at least $12 \mu\text{m}$ from each edge were analyzed for phalloidin or DNase I fluorescence pixel intensity per square micrometer using MetaMorph Imaging System (Molecular Devices Corp.).

Experimental Protocols

In the first set of experiments (n=10), mounted and pressurized arteries were equilibrated for 1 hour at 25 mm Hg. Pressure was increased to 125 mm Hg in increments of 25 mm Hg and lumen diameter measured at each pressure once stable. ONOO^- (10^{-4} mol/L) was added to the bath and lumen diameter measured. Each pressure step was repeated in the presence of ONOO^- to assess the affect on myogenic reactivity. Control vessels underwent both sets of pressure steps without addition of ONOO^- (vehicle control=Hepes PSS without ONOO^-). In the next experiments (n=7), an ONOO^- concentration response was obtained by cumulative addition of ONOO^- (10^{-8} to 10^{-4} mol/L) at a constant pressure of 75 mm Hg and measuring lumen diameter at each pressure. At the end of each experiment, papaverine (10^{-4} mol/L) was added to obtain fully relaxed diameters at pressures of 0 to 125 mm Hg. From these passive diameters, percent tone and dilation were calculated.

Drugs and Solutions

Hepes PSS was prepared fresh before each experiment and composed of (in mmol/L): 142 NaCl, 4.69 KCl, 1.17 MgSO_4 , 0.5 EDTA,

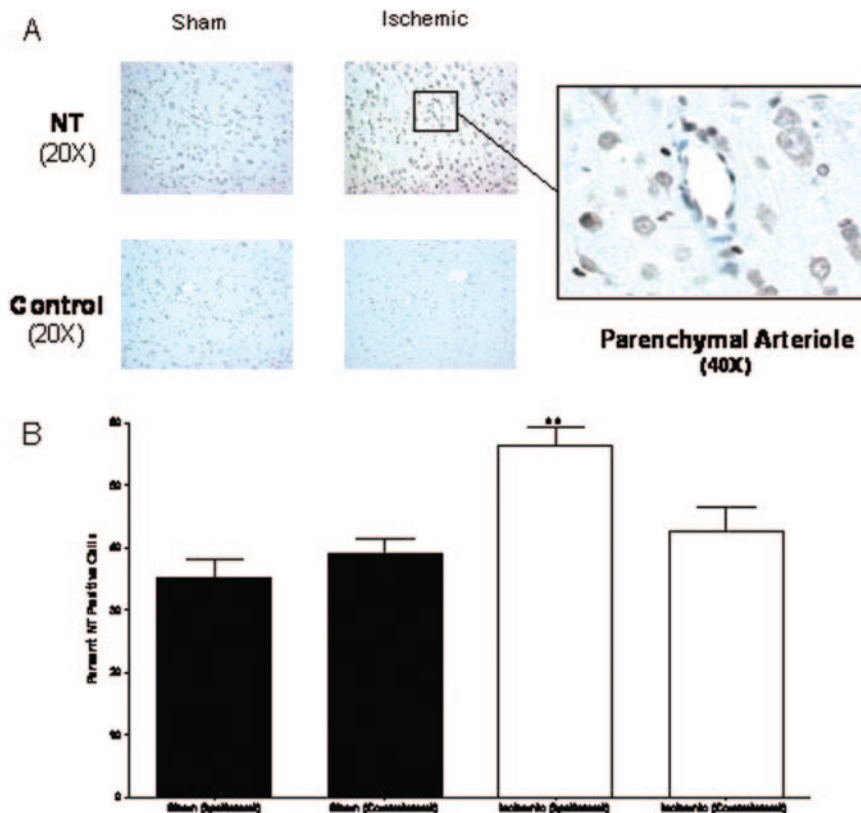


Figure 1. A, Photomicrograph of brain sections stained for NT (brown) and hematoxylin (blue) after 1 hour of ischemia by MCAO and 30 minutes of reperfusion. Control and sham brains showed modest NT staining, whereas the ischemic brains showed significant positive staining. Inset shows a $40\times$ image of an arteriole within the ischemic brain with vascular cells positive for NT (n=3 animals per group). B, Graph showing the percent of NT-positive vascular cells in brain sections from MCAO-induced I/R compared with controls. The ischemic brains showed a significant increase in NT-positive vascular cells compared with sham and contralateral sections (n=12 vessels per group per side; $**P < 0.01$ vs ipsilateral sham).

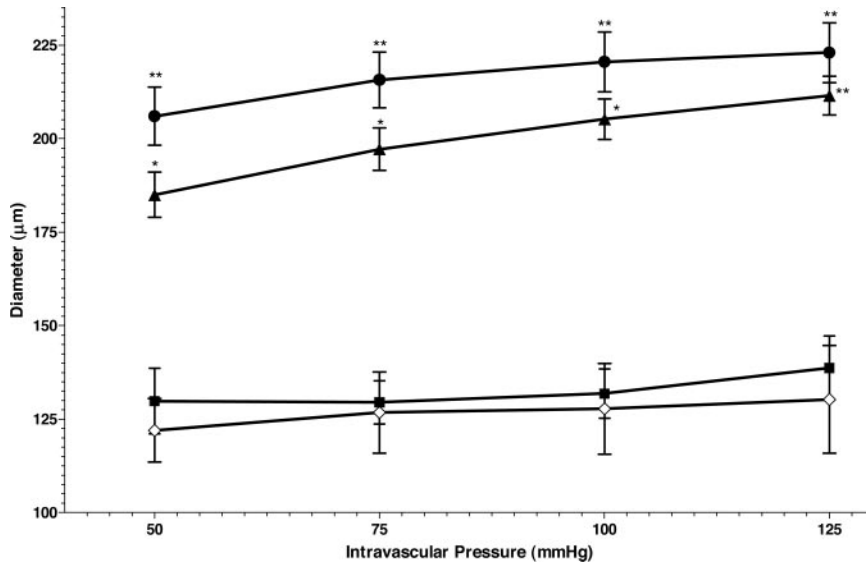


Figure 2. Graph showing diameter of PCAs after step increases of intravascular pressure from 50 to 125 mm Hg in the absence and presence of ONOO⁻ (10⁻⁴ mol/L). Vehicle controls underwent consecutive pressure steps without addition of ONOO⁻. The active response to pressure for each vessel was determined before addition of any drug. ■ indicates active response; ▲, ONOO⁻ (10⁻⁴ mol/L); ●, papaverine (10⁻⁴ mol/L); ◇, vehicle control (n=6 animals per group; *P<0.05, **P<0.01 vs active response).

2.79 CaCl₂, 10.0 Hepes, 1.2 KH₂PO₄, and 5.0 glucose. ONOO⁻ was purchased from Calbiochem and stored at -80°C. Papaverine was purchased from Sigma Chemical Co. All drugs were mixed as stock solutions (10⁻³; 10⁻⁴ mol/L) before each experiment.

Data Calculations and Statistical Analysis

Percent NT-positive cells was calculated as: $[\#_{\text{brown}}/(\#_{\text{brown}} + \#_{\text{blue}})] \times 100\%$, where $\#_{\text{brown}}$ was the number of NT-positive cells and $\#_{\text{blue}}$ was the number of negative NT cells.

Percent tone was calculated as the percent decrease in diameter from the relaxed diameter in papaverine at each pressure by the equation $[1 - (\phi_{\text{tone}}/\phi_{\text{papaverine}})] \times 100\%$, where ϕ_{tone} was diameter of the artery with tone, and $\phi_{\text{papaverine}}$ was the diameter in papaverine. Percent dilation was calculated as: $[1 - (\phi_{\text{ONOO}^-}/\phi_{\text{papaverine}})] \times 100\%$.

Data are presented as mean ± standard error. Differences in NT staining, percent tone, and percent dilation were determined by one-way ANOVA followed by Student-Newman-Keuls post hoc test for multiple comparisons where appropriate and considered significant at P<0.05.

Results

NT Positive Vascular Cells After I/R

Figure 1A shows a photomicrograph of brain sections from control, sham, and ischemic animals demonstrating NT-stained vessels. NT-positive staining (brown) is shown in the ischemic section, with considerably less staining in the sham. The high-power image (40×) of the ischemic section shows NT-positive cells localized in an arteriole. Figure 1B shows a graph of the percentage of NT-positive vascular cells after MCAO. Ischemic animals had a significantly greater percentage of NT-positive vascular cells compared with the sham control brains. The percent positive NT-stained vascular cells for ischemic versus sham ipsilateral and contralateral was 56±3% and 43±4% versus 35±3% and 39±3%; P<0.01.

Effect of ONOO⁻ on Myogenic Tone and Reactivity of PCAs

The diameter of PCAs after step increases from 50 to 125 mm Hg in the absence and presence of ONOO⁻ compared with the response in papaverine is shown in Figure 2. In the absence of ONOO⁻, increasing pressure caused little change in diameter, indicating active myogenic reactivity.

The addition of ONOO⁻ caused dilation and diminished myogenic reactivity, demonstrated by an increase in diameter as pressure was increased. In fact, the diameter response in the presence of ONOO⁻ was similar to that of papaverine. In a separate group of vessels, repeated pressure steps in the absence of ONOO⁻ did not alter PCA tone or reactivity to pressure, indicating that diminished myogenic activity was attributable to addition of ONOO⁻.

Figure 3 shows the percent tone at 75 mm Hg in the absence and presence of ONOO⁻. In the absence of ONOO⁻, the tone of PCAs was 43±5%, whereas addition of ONOO⁻ caused dilation, diminishing tone to 12±5% (P<0.01).

Concentration Response to ONOO⁻

The concentration response of PCAs to ONOO⁻ at 75 mm Hg is shown in Figure 4. At low concentrations (10⁻⁸ to 10⁻⁶ mol/L), addition of ONOO⁻ resulted in a decrease in lumen diameter from 129±16 to 115±15 (P<0.05). At a concentration of 6×10⁻⁶ mol/L, the vessels dilated significantly. Diameter did not change with increasing concentrations after this dilation. Additionally, no change in diameter was ob-

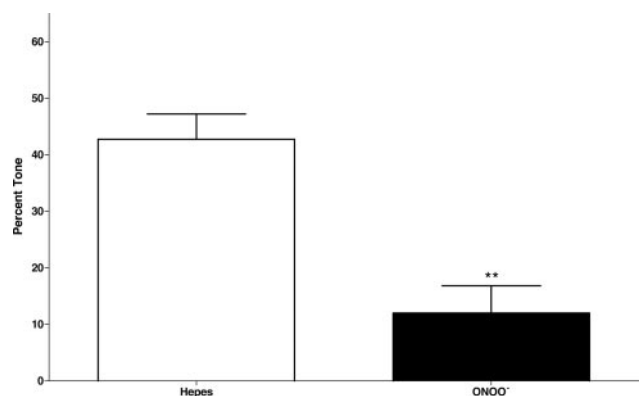


Figure 3. Graph showing percent tone of PCAs in the absence (open bar) and presence (closed bar) of ONOO⁻ (10⁻⁴ mol/L) at 75 mm Hg. The amount of tone was significantly diminished in the presence of ONOO⁻ (n=6 animals per group; **P<0.01 vs Hepes).

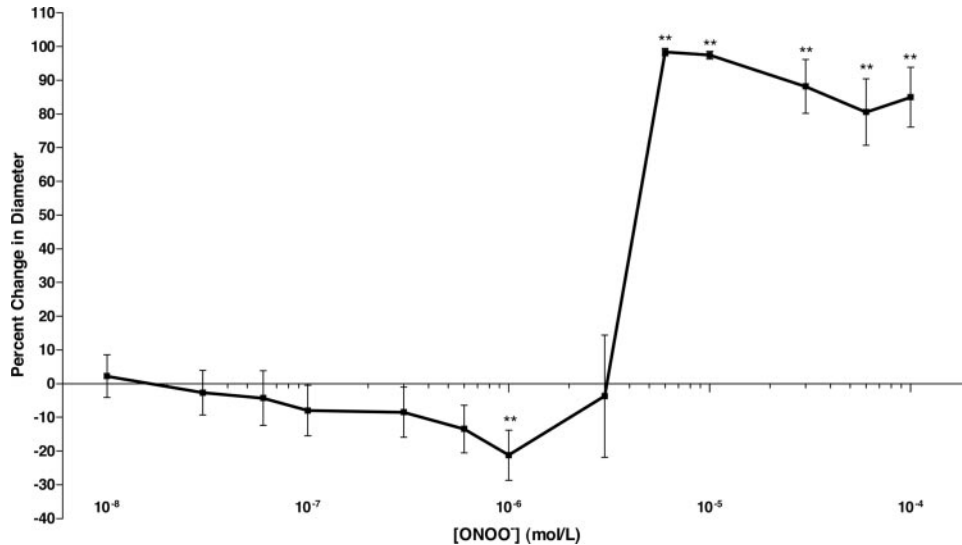


Figure 4. Graph showing PCA concentration response to cumulative addition of ONOO⁻ (10⁻⁸ to 10⁻⁴ mol/L) at 75 mm Hg. Addition of ONOO⁻ caused constriction at low concentrations (10⁻⁶ mol/L); however, the amount of tone significantly decreased in the presence ONOO⁻ at concentrations >6×10⁻⁶ mol/L (n=7; *P<0.05; **P<0.01 vs no ONOO⁻).

served in PCAs denuded of endothelium in response to ONOO⁻, indicating this was an endothelium-independent dilatation (data not shown).

Effect of ONOO⁻ on VSM F-Actin and G-Actin Content of PCAs

Figure 5 shows both F-actin and G-actin content of VSM from PCAs fixed under pressure in the absence and presence of ONOO⁻. As shown in Figure 5A through 5C, the presence of ONOO⁻ resulted in a 4.5-fold decrease in F-actin content compared with PCAs with spontaneous tone in the absence of ONOO⁻. Additionally, there was a 27% increase in G-actin content, determined by DNase I staining (Figure 5D). Therefore, addition of ONOO⁻ resulted in decreased F-actin and increased G-actin content in VSM of PCAs, indicating significant actin depolymerization.

Discussion

In the present study, we investigated the effect of ONOO⁻ on the structure and function of the cerebral vasculature and showed that low concentrations of ONOO⁻ resulted in constriction of PCAs, whereas higher concentrations caused vasodilation and loss of myogenic reactivity. In addition, ONOO⁻-induced dilation was associated with a decrease in VSM F-actin and an increase in G-actin content, demonstrating that ONOO⁻ caused actin depolymerization. Because immunohistochemical staining showed increased NT staining in the cerebrovasculature after MCAO with reperfusion, it is possible that nitrosylation of actin by ONOO⁻ affects its polymerization state, as shown previously.¹¹ Together, these findings suggest that ONOO⁻ damages the cerebrovasculature, resulting in decreased VSM F-actin and diminished myogenic activity.

A dynamic actin cytoskeleton, capable of polymerization and depolymerization, has been shown to be necessary for proper pressure-induced myogenic reactivity of cerebral VSM.^{5,6} In the present study, ONOO⁻-induced loss of

myogenic reactivity was associated with decreased VSM F-actin and increased G-actin content, suggesting that the loss of myogenic reactivity may be attributed to its effect on VSM actin. Additionally, previous studies have shown that diminished myogenic tone resulting from I/R injury was associated with a loss of F-actin in VSM similar to what was demonstrated in the present study.⁴ Because ONOO⁻ was increased in the cerebral vasculature after I/R, it is possible that the increased levels of ONOO⁻ produced during I/R may cause diminished myogenic activity through nitrosylation and depolymerization of VSM actin. In support of this, ONOO⁻ has been reported previously to inhibit polymerization of G-actin and induce depolymerization of F-actin in vitro.¹¹

The effect of I/R and ONOO⁻ on myogenic reactivity may impact cerebral blood flow autoregulation because myogenic reactivity is an underlying contributor to autoregulation.¹⁶ Autoregulation of cerebral blood flow has been shown to be disrupted during I/R,¹⁷ which can lead to blood-brain barrier disruption, edema formation, and brain tissue damage.¹⁸⁻²⁰ In the present study, we showed that ONOO⁻ was increased in the vasculature during I/R by immunohistochemical staining of NT. Because of its effect on myogenic tone and reactivity, increased ONOO⁻ could be a mechanism by which autoregulation is disrupted after I/R.²¹ Unregulated blood flow and decreased cerebrovascular resistance may lead to edema formation and blood-brain barrier disruption.^{18,20} Therefore, ONOO⁻-induced loss of myogenic activity may significantly contribute to brain injury after I/R.

Other studies have also shown an effect of ONOO⁻ on myogenic reactivity. Dewitt et al²² showed that addition of ONOO⁻ to isolated rat MCAs resulted in diminished vasodilatory responses to decreasing pressures and that this effect was attenuated by inhibition of ATP-dependent K⁺ channels. Additionally, Wei et al²³ showed that ONOO⁻ activated cat cerebral artery K⁺ channels through in vivo application of the ATP-sensitive K⁺ channel inhibitor glyburide. Other

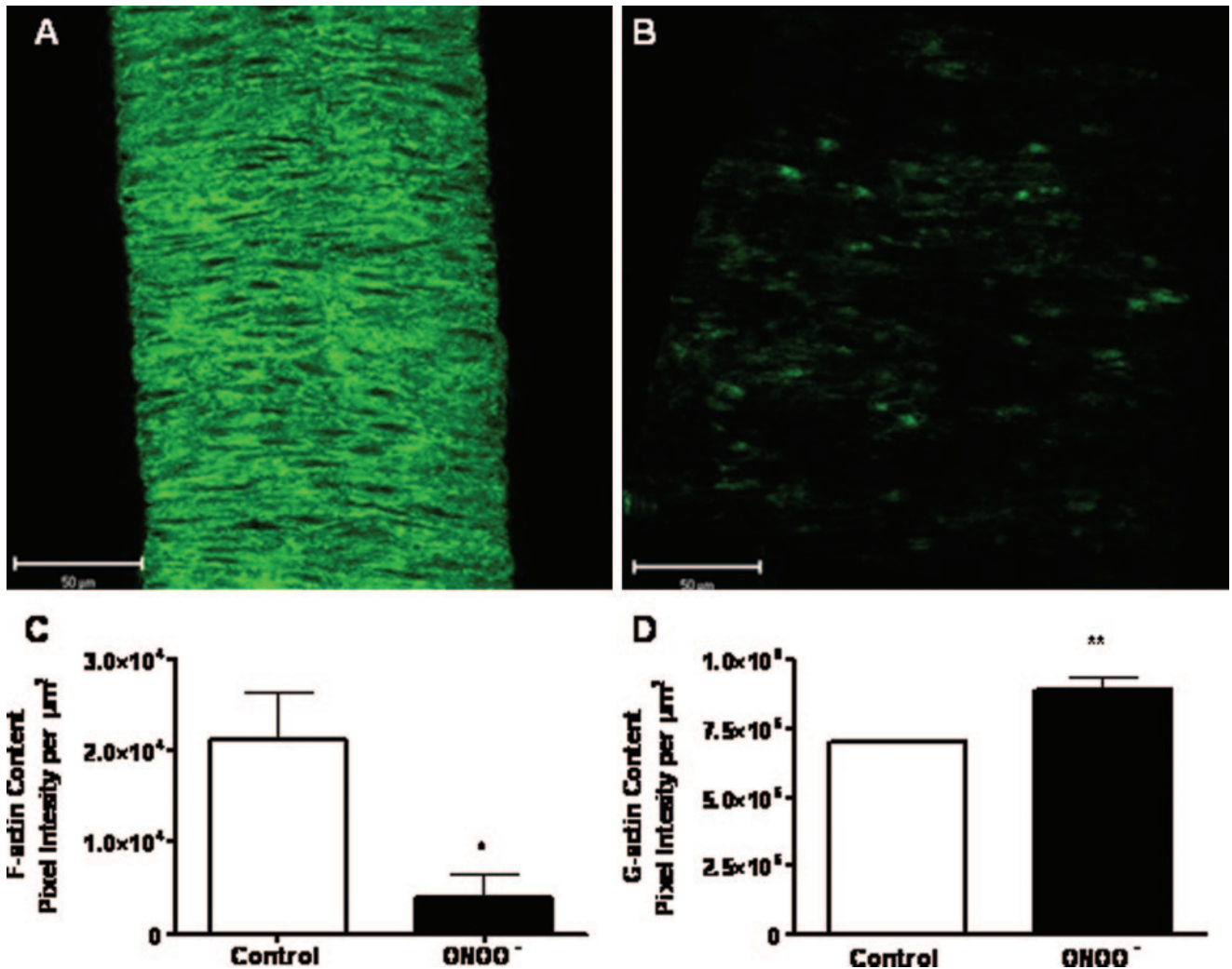


Figure 5. Photomicrographs showing F-actin content in pressurized PCAs with spontaneous tone (A) and in the presence of 10^{-4} mol/L ONOO^- (B) using phalloidin Oregon-Green. C, F-actin pixel intensity per μm^2 in pressurized PCAs with tone (control) and in 10^{-4} mol/L ONOO^- ($n=8$ per group). D, G-actin pixel intensity per μm^2 in pressurized PCAs with tone (control) and in 10^{-4} mol/L ONOO^- using DNase I, Alexa 488 ($n=5$ per group; * $P<0.05$, ** $P<0.01$ vs control).

studies also showed ONOO^- -induced dilation of canine cerebral artery rings and human coronary arterioles through hyperpolarization via K^+ channel activation and inhibition of membrane Ca^{2+} channels.^{12,23–25} In the present study, we cannot discount initial ONOO^- -induced vasodilation through these mechanisms. However, the effect of ONOO^- on the actin cytoskeleton of VSM suggests significant vascular damage that may impact stroke outcome.

The present study also showed constriction of cerebral arteries to low concentrations of ONOO^- , consistent with other reports showing increased tone in pulmonary and cerebral arteries using similar concentrations of ONOO^- .^{26,27} Both constriction and dilation of cerebral arteries induced by ONOO^- could potentially cause further injury during I/R. For example, constriction of cerebral vessels could increase the extent of ischemia, whereas dilation could cause a decrease in cerebrovascular resistance, promoting edema and blood–brain barrier disruption.^{18,19,28,29} Therefore, either effect of ONOO^- on myogenic tone and reactivity could promote further damage during stroke.

Although we did not measure the concentration of ONOO^- in the MCAO model in vivo, Dewitt et al proposed that the levels of ONOO^- during cerebral I/R may reach micromolar concentrations.²² This is based on the concentrations of NO and O_2^- during I/R measured in vivo and the rate kinetics of NO and O_2^- reactivity.^{7,30} Regardless of the current difficulty in the direct measurement of ONOO^- in vivo, the ONOO^- -induced reduction in F-actin content of VSM observed is similar to the loss of F-actin we reported previously to be associated with MCAO I/R.⁴ With the increase in ONOO^- and nitrosylation in the cerebrovasculature after I/R, these findings suggest that ONOO^- may be present in sufficient concentrations, similar to the concentration range used in this study, to cause depolymerization of VSM actin and subsequent loss of myogenic activity.

In summary, addition of ONOO^- to isolated and pressurized PCAs resulted in increased tone at low concentrations and diminished myogenic tone and loss of reactivity at high concentrations. ONOO^- -induced dilation was also associated with decreased F-actin and increased G-actin content of VSM

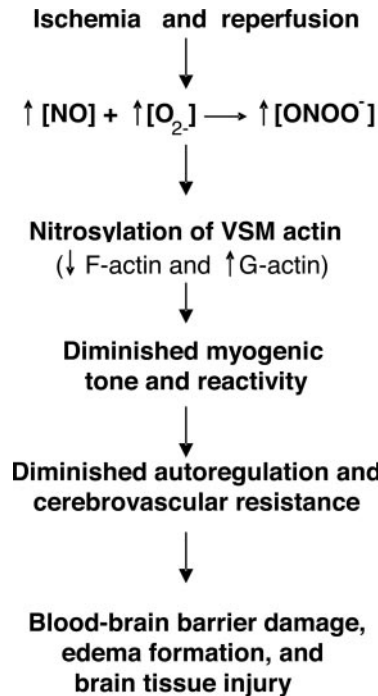


Figure 6. Hypothesized effect of ONOO^- on myogenic tone and reactivity via nitrosylation of VSM actin after I/R.

similar to the diminished F-actin content of VSM associated with I/R.⁴ We therefore hypothesize that increased ONOO^- production during I/R causes loss of myogenic activity through direct damage to the VSM actin cytoskeleton. The consequence of this is that it can lead to loss of autoregulation and decreased cerebrovascular resistance, resulting in additional brain tissue damage beyond the initial ischemic event. This hypothesis is outlined in Figure 6. This study implicates the possible effectiveness of O_2^- and ONOO^- scavengers targeted to the cerebrovasculature as a mechanism of both vascular and neuroprotection to damage after I/R.³¹

Acknowledgments

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