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Peroxynitrite diminishes myogenic tone in cerebral arteries: role of nitrotyrosine and F-actin

Matthew J. Maneen and Marilyn J. Cipolla

Department of Neurology, University of Vermont, Burlington, Vermont

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Maneen MJ, Cipolla MJ. Peroxynitrite diminishes myogenic tone in cerebral arteries: role of nitrotyrosine and F-actin. *Am J Physiol Heart Circ Physiol* 292: H1042–H1050, 2007. First published October 13, 2006; doi:10.1152/ajpheart.00800.2006.—This study investigated the effect of peroxynitrite (ONOO⁻)-induced nitrosylation of filamentous (F)-actin on myogenic tone in isolated and pressurized posterior cerebral arteries (PCAs). Immunohistochemical staining was used to determine 3-nitrotyrosine (NT) and F-actin content in vascular smooth muscle after exposure to 10⁻⁷ M or 10⁻⁴ M ONOO⁻ for 5 or 60 min in isolated third-order PCAs (*n* = 37) from male Wistar rats pressurized to 75 mmHg in an arteriograph chamber, quantified with confocal microscopy. Additionally, the role of K⁺ channels in ONOO⁻-induced dilation was investigated with 3 μM glibenclamide or 10 mM tetraethylammonium chloride before ONOO⁻ exposure. ONOO⁻ (10⁻⁴ M) induced a 40% dilation of tone (*P* < 0.05) while diminishing F-actin content by half (*P* < 0.05) and causing a 60-fold increase in NT (*P* < 0.05) in the vascular smooth muscle of PCAs. Additionally, F-actin was inversely correlated with both diameter and NT content (*P* < 0.05) and was significantly colocalized in the vascular smooth muscle with NT (overlap coefficient = 0.8). The dilation to ONOO⁻ was independent of K⁺ channel activity and thiol oxidation as glibenclamide, tetraethylammonium chloride, and dithiothreitol had no effect on ONOO⁻-induced dilation or F-actin or NT content in PCAs. Because NT was colocalized with F-actin, we hypothesize that ONOO⁻ induces nitrosylation of F-actin in vascular smooth muscle leading to depolymerization and the subsequent loss of myogenic tone, which may promote vascular damage during oxidative stress such as in ischemia and reperfusion injury.

oxidative stress; cerebrovasculature; myogenic activity; actin cytoskeleton

PEROXYNITRITE (ONOO⁻) is a relatively stable reactive nitrogen and oxygen species, produced from the interaction of nitric oxide and superoxide, capable of diffusing through phospholipid membranes (5, 40). We showed previously (39) that exposure to exogenous ONOO⁻ results in dilation of spontaneous tone and loss of myogenic reactivity in isolated and pressurized rat third-order posterior cerebral arteries (PCAs). Additionally, ONOO⁻-induced dilation was associated with a loss of vascular smooth muscle (VSM) filamentous (F)-actin similar to that seen after cerebral ischemia with reperfusion (12, 14, 39). This is in agreement with other studies that have shown that ONOO⁻ can induce both depolymerization of F-actin and inhibition of G-actin polymerization (16, 51). Since a dynamic actin cytoskeleton is a significant contributor to pressure-induced myogenic activity (13, 15), production of ONOO⁻ may have a deleterious effect on cerebrovascular resistance through disruption of VSM actin following condi-

tions of increased oxidative stress. This is of importance since the loss of myogenic activity in cerebral resistance arteries, as in cerebral ischemia and reperfusion injury, results in increased and unregulated intravascular pressure on distal capillary beds, which may lead to blood-brain barrier disruption and edema formation (32, 53).

The mechanisms by which ONOO⁻ results in the loss of myogenic activity and diminished F-actin have yet to be elucidated. ONOO⁻ has been shown to nitrosylate tyrosine residues on proteins, including actin cytoskeletal proteins (5, 16, 22, 25, 40). We therefore hypothesized that ONOO⁻-induced nitrosylation of actin protein results in depolymerization of VSM F-actin and is one mechanism that may lead to a loss of myogenic tone and reactivity to changes in intravascular pressure. In the present study, we determined the 3-nitrotyrosine (NT) and F-actin content in VSM from PCAs exposed to various concentrations of ONOO⁻. The increased nitration of actin tyrosine residues may be one mechanism by which the contractile properties of VSM are disrupted after nitrosative and oxidative stress. We therefore also investigated the degree of NT colocalization with F-actin in the VSM of PCAs dilated with ONOO⁻. In addition, since previous studies have reported that ONOO⁻ can affect K⁺ channel activity, causing hyperpolarization of VSM (35, 36, 43, 56), we also determined whether K⁺ channels are a component of ONOO⁻-induced dilation in PCAs.

MATERIALS AND METHODS

Animals. Male Wistar rats (350–450 g; Harlan) were used for all experiments. Animals were housed in the University Animal Care Facility, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. Food and water were supplied ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee.

Pressurized arteriograph system and PCA preparation. Animals were anesthetized with isoflurane and decapitated, and the brain was quickly removed. Third-order PCAs (100- to 120-μm inner diameter with tone) were carefully dissected, mounted on glass cannulas in an arteriograph chamber, and pressurized to 75 mmHg. PCAs were used for all investigations because they develop consistent and significant myogenic tone and reactivity to pressure (45). The Living Systems Instrumentation arteriograph chamber was connected to both a 100-ml reservoir, which allowed for the addition of drugs, and a heat exchanger that recirculated HEPES-buffered physiological saline solution (HEPES-PSS) at a constant temperature of 37 ± 0.5°C. The distal cannula was closed off to prevent luminal flow, and experimental pressure was maintained at 75 mmHg with a pressure servo controller and a peristaltic pump. Intravascular diameter was measured with a video dimension analyzer, and both diameter and pressure tracings

Address for reprint requests and other correspondence: M. J. Cipolla, Univ. of Vermont, Dept. of Neurology, 89 Beaumont Ave., Given C454, Burlington, VT 05405 (e-mail: marilyn.cipolla@uvm.edu).

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were recorded with WinDaq Acquisitions software (DATAQ Instruments).

Experimental protocol. To determine the relationship between lumen diameter, F-actin content, and NT, the same vessels were used for all three measurements. Briefly, mounted and pressurized PCAs were equilibrated at 75 mmHg, and lumen diameter was measured after spontaneous tone was acquired. PCA lumen diameter was measured after 5 or 60 min of exposure to different concentrations of ONOO⁻ (10^{-7} M or 10^{-4} M) ($n = 5$ per time per concentration) and then fixed while pressurized by addition of 37% formaldehyde directly to the 20-ml HEPES within the arteriograph chamber for 30 min for subsequent staining for F-actin and NT. Additionally, another set of vessels ($n = 3$) was exposed to 10^{-7} M ONOO⁻ every 10 min for 60 min, and lumen diameter was measured before fixation. Control vessels (i.e., degraded ONOO⁻, papaverine, cytochalasin D) were fixed after equal time points in the absence of ONOO⁻. Degraded ONOO⁻ was left at room temperature for 1 h with no protection from direct light and was added in place of ONOO⁻ for 1 h at 10^{-4} M ($n = 3$). Cytochalasin D (10^{-5} M; $n = 3$) and papaverine (10^{-4} M; $n = 3$) were utilized in place of ONOO⁻ for 1 h as negative controls for dilation both with and without loss of F-actin, respectively.

To investigate the involvement of K⁺ channels in mediating dilation to ONOO⁻, PCAs were incubated in 10 mM tetraethylammonium chloride (TEA; $n = 3$) or 3 μ M glibenclamide ($n = 3$) before

exposure to 10^{-4} M ONOO⁻ and lumen diameter was measured at 5, 10, 30, and 60 min. Vessels were then fixed, stained, and analyzed for F-actin and NT content.

To exclude the possibility that thiol oxidation is involved in ONOO⁻-induced dilation and F-actin and NT content, PCAs ($n = 4$) pressurized to 75 mmHg with spontaneous myogenic tone were incubated with the thiol reductant dithiothreitol (1 mM) 5 min before exposure of ONOO⁻ (10^{-4} M for 1 h) and remained in the bath for the entirety of the experiment. PCA diameter was measured with tone, 5 min after dithiothreitol, and 1 h after ONOO⁻ in the presence of dithiothreitol. Vessels were then fixed while pressurized and stained for F-actin and NT as described below.

F-actin and NT staining and quantification. PCAs that were fixed while pressurized at 75 mmHg were subsequently whole-mount double stained for F-actin with phalloidin-Oregon Green 488 (Molecular Probes) and for NT with a mouse anti-NT primary antibody (Upstate) at 1:200 and a goat anti-mouse IgG-conjugated Alexa Fluor 568 (Molecular Probes) secondary antibody at 1:800, using established techniques (13, 14, 39). Arteries were imaged with a Zeiss LSM 510 laser scanning confocal microscope at $\times 40$. The advantage of confocal microscopy is the ability to obtain thin optical sections and eliminate error such as thick, out-of-focus fluorescence images (55). Optimal gain for F-actin staining was based on PCAs with spontaneous tone at 75 mmHg in the absence of ONOO⁻, and NT gain was

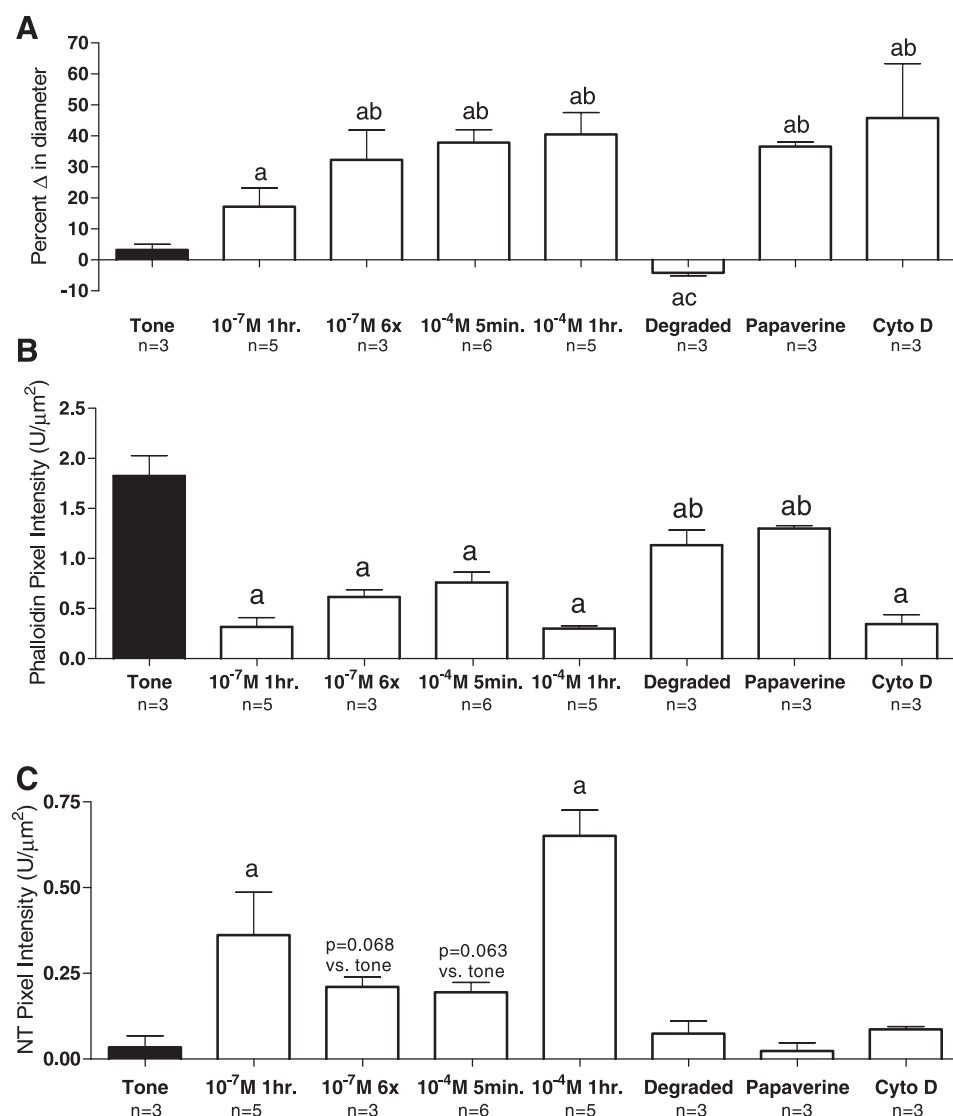


Fig. 1. Effect of peroxynitrite (ONOO⁻) on cerebral artery diameter, filamentous (F)-actin, and 3-nitrotyrosine (NT) content. **A:** % change in diameter of pressurized (75 mmHg) posterior cerebral arteries (PCAs) in the absence (Tone) and presence of ONOO⁻. Single ONOO⁻ exposures at 10^{-4} M for 5 min and 1 h and at 10^{-7} M every 10 min for 60 min are presented. Degraded ONOO⁻ (Degraded), papaverine (10^{-4} M), and cytochalasin D (10^{-5} M; Cyto D) were used as controls in the absence of peroxynitrite. **B:** F-actin pixel intensity per μ m² in vascular smooth muscle (VSM) in the same vessels as in **A**, determined with phalloidin-Oregon Green pixel intensity per μ m² in each group. **C:** NT pixel intensity per μ m² in VSM in the same vessels as in **A** and **B**, determined with an anti-NT antibody with an Alexa Fluor 568 secondary (^a $P < 0.05$ vs. Tone; ^b $P < 0.05$ vs. a; ^c $P < 0.05$ vs. a and b).

standardized with PCAs treated with 10^{-4} M ONOO⁻ and stained with the secondary antibody only. Confocal LSM images for the emission spectra 488 and 568 nm were scanned sequentially for each channel to eliminate fluorophore cross talk and obtain reliable images for quantification of colocalization. Every image was taken with the same laser and LSM setting including the gain for each fluorophore channel and remained constant for all arteries analyzed. Each vessel was quantified for both NT and F-actin content after analysis of diameter in each artery, as well as colocalization (described below).

Images ($\times 40$) focused on the VSM at a depth of 1.5 μm from the abluminal surface and centered on the artery at least 12 μm from each edge were then analyzed for phalloidin and NT fluorescence pixel intensity per square micrometer (units/ μm^2) with MetaMorph Imaging Systems (Molecular Devices) (39). Additionally, phalloidin and NT colocalization in VSM was determined with Zeiss LSM Meta Software (Carl Zeiss) (11, 26). Colocalization was characterized by the degree of overlap between two channels in a microscopy image. The overlap coefficient is a generally accepted measure of colocalization. It indicates channel signal overlap and represents the true degree of colocalization (11, 33). Although colocalization is relative, analysis utilizing mathematical coefficients such as the overlap coefficient provides much more quantitative justification than evaluation by naked eye (59). The overlap coefficient was calculated with the above software and is a product of channel intensities that return a significant value only when both channel values belong to the same pixel. This value ranges between 0 and 1 and is not sensitive to variations in fluorophore intensities. A value ≥ 0.7 was considered colocalized and implies that 70% of both objects overlap with the other object (11). Background and noise were removed by adjusting the threshold value for each channel displayed by each histogram, from which the overlap coefficient was calculated and remained constant for each artery.

Drugs and solutions. HEPES-PSS was prepared fresh before each experiment and contained (mM) 142 NaCl, 4.69 KCl, 1.17 MgSO₄, 0.5 EDTA, 2.79 CaCl₂, 10.0 HEPES, 1.2 KH₂PO₄, and 5.0 glucose. ONOO⁻ was purchased from Calbiochem and stored at -80°C . During each experiment, ONOO⁻ was kept on ice, and direct exposure to sunlight was prevented. ONOO⁻ was degraded by sitting at room temperature for 1 h in a clear container. Cytochalasin D was purchased from Calbiochem, and papaverine, glibenclamide, and TEA were purchased from Sigma. All drugs were mixed as stock solutions before each experiment.

Data calculations and statistical analysis. Results are presented as means \pm SE. The *n* value represents the number of vessels in each treatment group. All statistical analysis was determined with one-way analysis of variance (ANOVA) where appropriate, followed by Student-Newman-Keuls post hoc test for multiple comparisons or repeated-measures ANOVA, and considered significant at $P \leq 0.05$. Linear regressions were calculated with GraphPad Prism software. Percent change in diameter was calculated as $[1 - (\phi_{\text{Tone}}/\phi_{\text{ONOO}^-}) \times 100]$, where ϕ_{Tone} is diameter of the artery at the start of the experiment with tone and ϕ_{ONOO^-} is the diameter after ONOO⁻ exposure.

RESULTS

ONOO⁻-induced dilation is associated with decreased F-actin. Figure 1A shows the percent change in diameter for all treatment groups. The addition of ONOO⁻ caused dilation in all treatment groups compared with both nontreated control (tone) and degraded ONOO⁻ control. This dilation was endothelium independent, because endothelial denudation of vessels did not alter the ONOO⁻-induced dilatation (unpublished results), as has been reported in other studies (35, 36, 60). A single exposure to 10^{-4} M ONOO⁻ resulted in maximum dilation within 5 min, and arteries remained maximally dilated

after 1 h. Both a single exposure and six exposures of 10^{-7} M ONOO⁻ resulted in a significant dilation and, as in the higher concentration, arteries did not return to basal tone within the 1-h experimental time period. Papaverine was used as a positive control for dilation independent of F-actin loss, whereas cytochalasin D was used as a control for dilation of tone with diminished F-actin. Both of these treatments resulted in significant dilation.

Figure 1B shows F-actin content in all treatment groups shown in Fig. 1A. In agreement with our previous findings (39), exposure to ONOO⁻ resulted in a significant loss of VSM F-actin as measured by phalloidin staining (Fig. 1B; see Fig. 3). At the highest concentration, ONOO⁻ (10^{-4} M) decreased F-actin content by half within 5 min, and content was further decreased after the 1-h incubation period. A single exposure of 10^{-7} M ONOO⁻ decreased F-actin similarly to the 10^{-4} M 1-h time point, whereas multiple exposures of 10^{-7} M over a 1-h time period did not have any further influence on VSM F-actin content than the single exposure. Cytochalasin D, which causes dilation and loss of F-actin (13), had both a dilatatory response and a diminished VSM F-actin content similar to the ONOO⁻ exposure groups. Papaverine also resulted in a decrease in F-actin compared with the tone control, although to a much lesser extent than with either ONOO⁻ or cytochalasin D.

Figure 1C shows the NT content in all groups of vessels. NT content was increased in VSM of PCAs exposed to ONOO⁻ (Fig. 1C). Exposure to 10^{-4} M ONOO⁻ for 1 h resulted in a 60-fold increase in VSM NT. Exposure to ONOO⁻ for 5 min increased NT as well ($P = 0.063$). Exposure to 10^{-7} M ONOO⁻ resulted in a significant elevation in NT; however, there was no significant change to repeated exposures at this lower concentration. NT content remained at basal levels in all

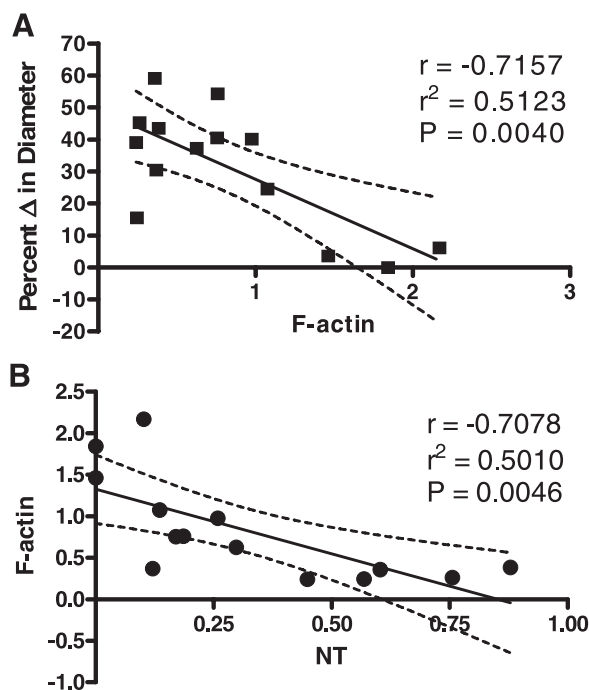


Fig. 2. Correlation and linear regression analysis between the changes in diameter vs. F-actin content (A) and F-actin content vs. NT content (B) in VSM of PCAs treated with ONOO⁻. Arteries with spontaneous tone and exposed to 10^{-4} M ONOO⁻ for 5 min or 1 h were used in the analysis ($n = 14$; $P < 0.01$).

four groups of controls (i.e., tone only, degraded ONOO⁻, papaverine, and cytochalasin D).

Loss of F-actin is correlated and colocalized with increased VSM NT. When all vessels were pooled, there was a significant inverse correlation ($r = -0.72$; $P < 0.01$) between ONOO⁻-induced dilation and VSM F-actin content (Fig. 2A). There was also a significant inverse correlation ($r = -0.71$; $P < 0.01$) between VSM F-actin and NT content of PCAs exposed to ONOO⁻ (Fig. 2B). To confirm this, F-actin and NT colocalization studies were done. There was significant colocalization of NT and F-actin in the VSM of PCAs exposed to ONOO⁻. Figure 3 shows confocal images of VSM F-actin and NT in PCAs and colocalization of vessels exposed to 10⁻⁴ M ONOO⁻ for 1 h. Arteries exposed to 10⁻⁴ M ONOO⁻ for 1 h had an overlap colocalization coefficient of 0.8 for NT and F-actin in VSM (Fig. 3), suggesting that ONOO⁻ induces nitrosylation of F-actin and may play a role in destabilization of F-actin. Taken together, these data support our previous hypothesis (39) that ONOO⁻-induced dilation of myogenic tone may be mediated by diminished VSM F-actin. Furthermore, the correlation between NT and F-actin suggests that nitrosylation of F-actin may be one mechanism by which ONOO⁻ causes diminished F-actin in VSM.

To exclude the possibility that ONOO⁻-induced dilation was due to thiol oxidation as opposed to nitrosylation, we determined ONOO⁻-induced dilation and VSM F-actin and NT content in PCAs after incubation in 1 mM dithiothreitol, a powerful thiol reductant (Fig. 4). Dithiothreitol had no effect on ONOO⁻-induced dilation, nor did it prevent the loss of F-actin and increased NT content in VSM, suggesting that ONOO⁻-induced dilation was not due to thiol oxidation.

ONOO⁻-induced dilation, diminished F-actin, and elevated NT content are independent of K⁺ channel activity. To exclude the possibility that ONOO⁻-induced dilation is due to the modulation of ATP-dependent (K_{ATP}) and/or Ca²⁺-dependent (BK) K⁺ channel activity as others have shown (1, 8, 21, 38, 43, 54), we used two established K⁺ channel inhibitors, glibenclamide and TEA. Glibenclamide was used to specifically inhibit K_{ATP} channels, whereas TEA was used as a nonspecific K⁺ channel inhibitor as previously reported (23, 30, 35, 37). Neither glibenclamide nor TEA had an effect on ONOO⁻-induced dilation (Fig. 5). Even though addition of TEA resulted in a significant increase in myogenic tone, exposure to ONOO⁻ still resulted in maximum dilation similar to non-TEA-treated controls. Although treatment with glibenclamide also resulted in an increase in tone, it was not statistically

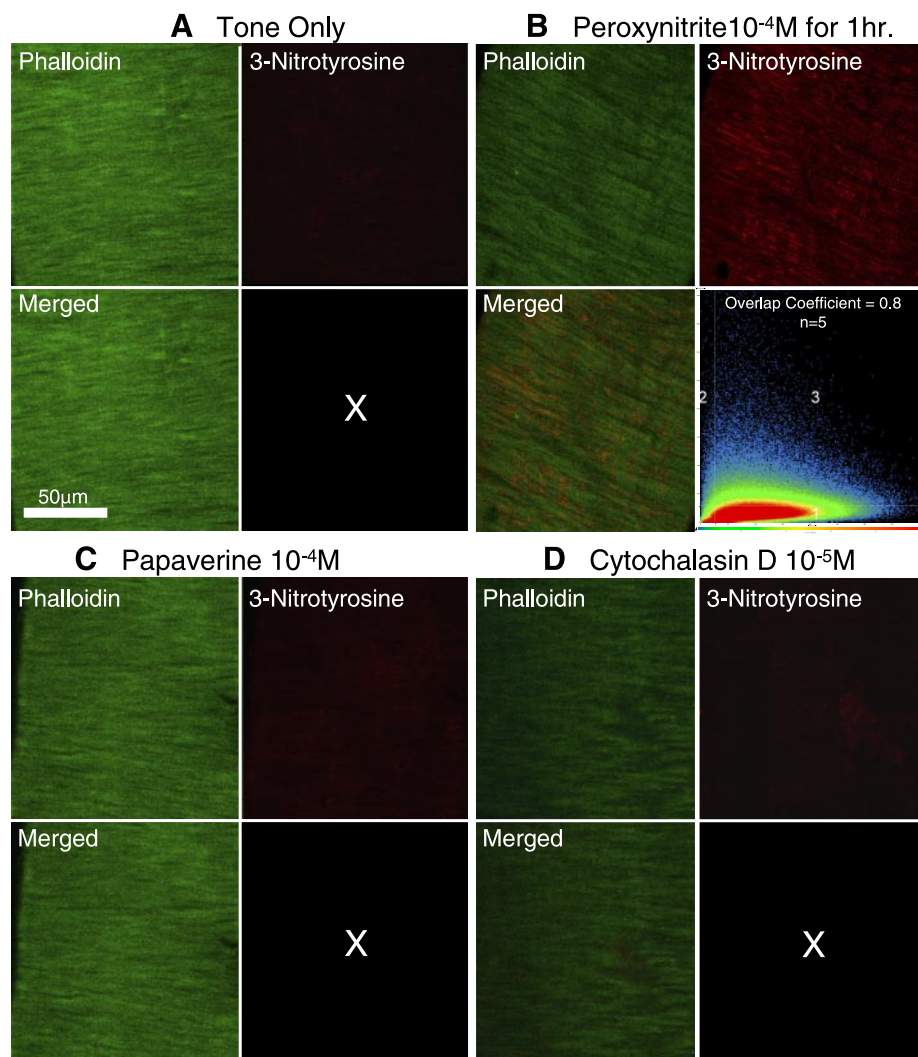


Fig. 3. Representative confocal images of PCAs pressurized to 75 mmHg with spontaneous myogenic tone were stained for both F-actin and NT, using phalloidin-Oregon Green and an anti-nitrotyrosine primary antibody with an Alexa Fluor secondary. A: control PCA with tone. B: PCA exposed to ONOO⁻ (10⁻⁴ M) for 1 h. C: PCA treated with papaverine (10⁻⁴ M). D: PCA treated with cytochalasin D (10⁻⁵ M). ONOO⁻ exposure (B) resulted in considerably less phalloidin staining than the control group and also showed increased NT staining. Colocalization of NT and F-actin is demonstrated in B by a representative histogram of F-actin vs. NT pixel intensity per µm² in VSM. Area 1 is F-actin, area 2 is NT, and area 3 is colocalized F-actin and NT (overlap coefficient = 0.8 ± 0.0; n = 5). Papaverine had phalloidin and NT staining similar to the control (Tone only) vessels. Additionally, treatment with cytochalasin D, a potent vasodilator that results in a significant loss of F-actin similar to exposure to ONOO⁻, showed no increase in NT staining but diminished F-actin content.

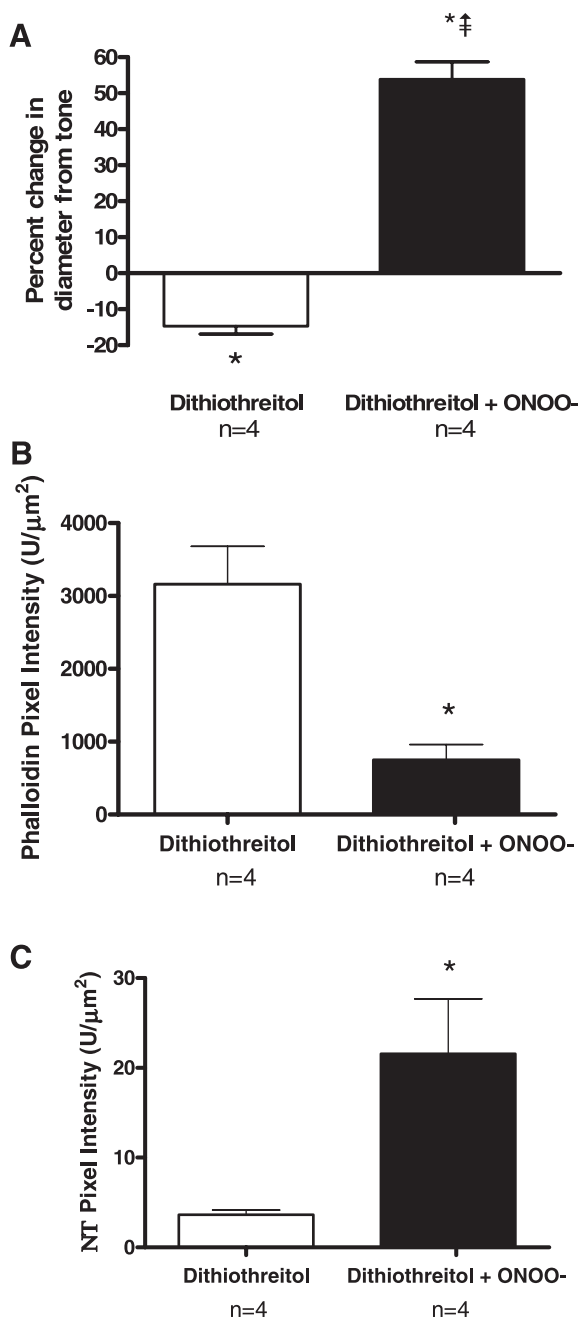


Fig. 4. Effect of the thiol reductant dithiothreitol on ONOO⁻-induced dilation and VSM F-actin and NT content. **A:** % change in diameter from the baseline diameter with tone in the presence of 1 mM dithiothreitol before addition of ONOO⁻. Although treatment with the reductant before ONOO⁻ exposure resulted in significant constriction of tone, it did not attenuate the ONOO⁻-induced dilation (**P* < 0.05 vs. tone; ‡*P* < 0.05 vs. dithiothreitol). **B:** VSM F-actin content of the vessels in **A**. The presence of dithiothreitol did not prevent the decrease in F-actin content induced by ONOO⁻ (**P* < 0.05 vs. without ONOO⁻). **C:** NT content in VSM of the arteries in **A** and **B**. Dithiothreitol had no effect on ONOO⁻-induced increase in VSM NT content (**P* < 0.05 vs. without ONOO⁻).

significant and did not alter the final diameter after exposure to ONOO⁻. Figure 5B shows the change in diameter of PCA in response to ONOO⁻ in the presence of either TEA or glibenclamide. There was no significant difference in diameter after ONOO⁻ treatment in all three groups, even though TEA

treatment initially constricted vessels significantly from tone. In addition, VSM F-actin and NT content were similar to those with 10⁻⁴ M ONOO⁻, resulting in diminished F-actin and increased NT in VSM (data not shown). These data suggest that ONOO⁻-induced dilation, increased VSM NT, and loss of VSM F-actin in rat PCAs is not dependent on K⁺ channel activity.

DISCUSSION

In the present study, we investigated the effect of ONOO⁻ on NT and F-actin content in VSM of PCAs and showed that ONOO⁻-induced dilation and diminished F-actin were associated with an increase in NT. Additionally, there was a significant correlation between F-actin and arterial dilation as well as NT content in PCA exposed to ONOO⁻. In fact, NT and F-actin were colocalized in VSM (Fig. 3), suggesting that nitrosylation of F-actin may be one mechanism by which ONOO⁻ causes diminished F-actin in the VSM and subsequent dilation of myogenic tone. This could impact disease states with increased blood perfusion and high oxygen or nitrate stress such as ischemia and reperfusion, traumatic brain injury, vasospasm, and hyperoxic seizures via detrimental effects on cerebral microvessels and blood flow (9, 53).

It is possible that another mechanism may be involved in the initial dilation to ONOO⁻ before its nitrosylation of actin. Since the loss of F-actin is in general a slow process, it further supports the possibility that ONOO⁻ causes dilation initially through a means other than diminished F-actin. Because others have shown K⁺ channel activity, specifically K_{ATP} and BK channels, to be involved in ONOO⁻-induced dilation of both peripheral and cerebral arteries via membrane hyperpolarization (8, 35, 43, 54), we investigated this possibility through specific and nonspecific inhibition of K⁺ channels. However, in this study, K_{ATP} and BK channel inhibition by glibenclamide and TEA, respectively, showed no effect on either ONOO⁻-induced dilation of tone or F-actin and NT content in PCAs, suggesting that ONOO⁻ acts independently of these K⁺ channels in PCA. This discrepancy may be due to regional differences in ONOO⁻-induced effects on K⁺ channels. In support of this, Ohashi et al. (43) showed that ONOO⁻-induced relaxation of rabbit internal carotid artery smooth muscle, but not the common carotid, is mediated by K_{ATP} channels. Therefore, it appears that regional differences in channel activity and density may play a role in modulating the effect of ONOO⁻ on myogenic activity in the cerebral vasculature. Although we investigated the role of K_{ATP} and BK channels, since their activity has been shown to be modulated by ONOO⁻ (8, 35, 43, 54), we cannot rule out the possible effect of other K⁺ channels such as inward rectifier (29, 46), voltage-gated (2, 10, 29, 31), and/or two-pore domain (7) K⁺ channels, which have been shown to dilate small cerebral arteries and have been implicated in myogenic activity. Currently, however, no studies have been completed in regard to these channels and possible modulation by ONOO⁻. Additionally, we cannot rule out the possibility that ONOO⁻ causes dilation through other means such as increased cGMP or modulation of intracellular Ca²⁺ through mechanisms such as increased sarcoplasmic reticulum Ca²⁺-ATPase channel activity (17, 54).

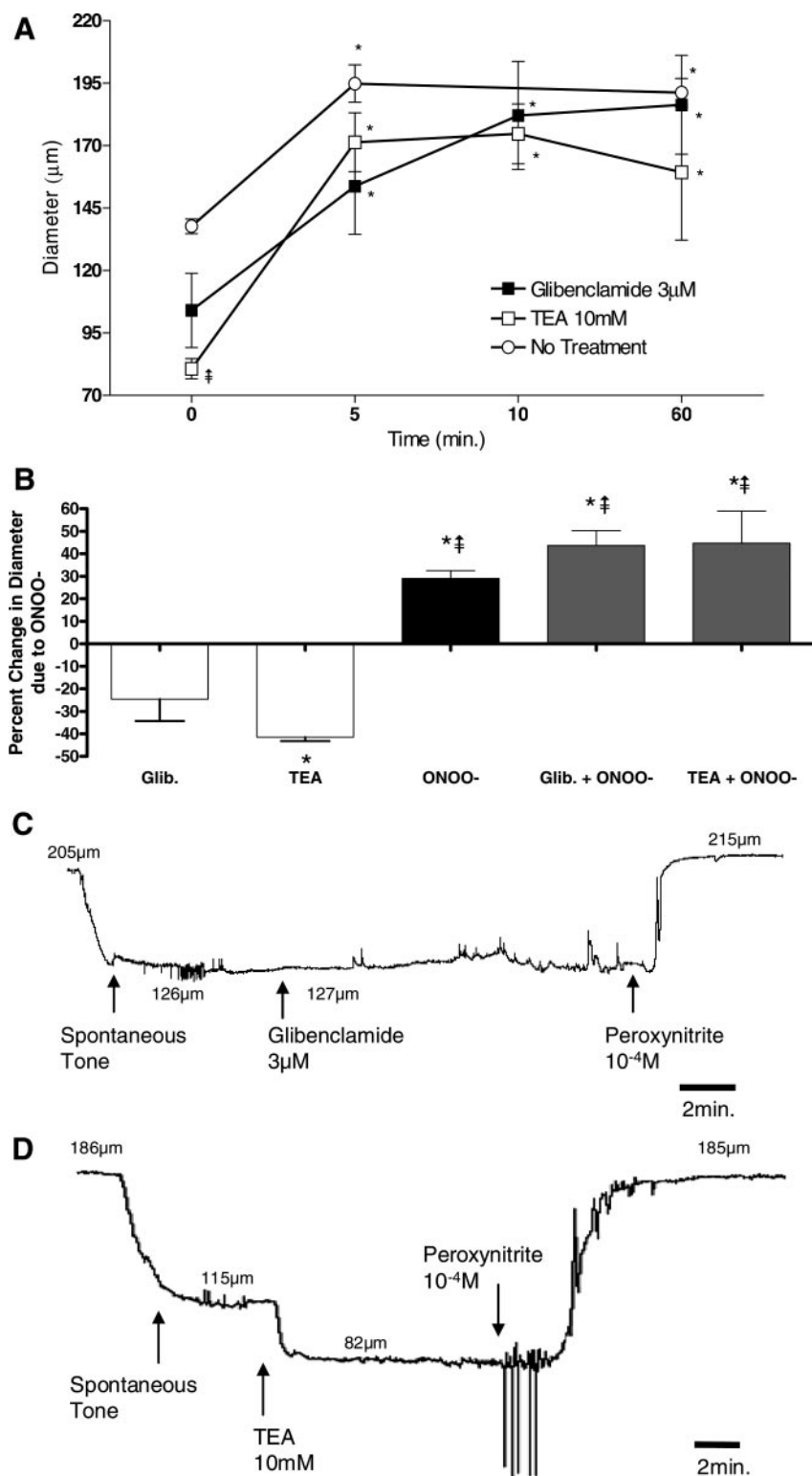


Fig. 5. Effect of K^+ channel inhibition on $ONOO^-$ -induced dilation of PCAs. **A**: average diameter of PCAs vs. time, pressurized to 75 mmHg and treated with glibenclamide or tetraethylammonium chloride (TEA) before $ONOO^-$ exposure. \circ , arteries in the absence of both glibenclamide and TEA ($n = 6$); \blacksquare , treated with 3 μ M glibenclamide ($n = 3$); \square , treated with 10 mM TEA ($n = 3$). All vessels were exposed to $ONOO^-$ (10^{-4} M) at the 0 time point. Treatment with K^+ channel blockers had no effect on the dilation to $ONOO^-$ within a similar time period. (* $P < 0.05$ vs. Tone; ‡ $P < 0.05$ vs. no treatment). **B**: % change in diameter from tone of the same arteries in **A** following K^+ channel inhibition and 10 min of $ONOO^-$ exposure. There was an increase in tone after exposure to glibenclamide and TEA; however, only TEA treatment was significantly different from the tone-only control. $ONOO^-$ exposure resulted in significant dilation from tone whether alone or in the presence of either inhibitor. There was no significant difference in % change in diameter after exposure to $ONOO^-$ in the presence of either TEA or glibenclamide inhibition of K^+ channels compared with $ONOO^-$ alone. Glib, 3 μ M glibenclamide; TEA, 10 mM TEA; Cont, control (no inhibitor). (* $P < 0.05$ vs. Tone; ‡ $P < 0.05$ vs. no $ONOO^-$). **C** and **D**: representative diameter tracings for each experiment showing that K^+ channel inhibition did not significantly affect the dilation to $ONOO^-$.

It also appears that although thiol oxidation plays a role in VSM relaxation due to other oxidants such as hydrogen peroxide (47), dilation due to $ONOO^-$ in pressurized PCAs acts independent of thiol oxidation, as determined through incubation with the thiol reductant dithiothreitol. Treatment with dithiothreitol resulted in an initial constriction of tone; however, it did not prevent dilation due to $ONOO^-$, which was

similar to the dilation observed in the absence of dithiothreitol (Figs. 1 and 4). In addition, $ONOO^-$ -induced loss of VSM F-actin and increased NT content in the presence of dithiothreitol were similar to those in the absence of the reductant. Finally, the reductant on its own did not induce F-actin loss or increase NT content in VSM. These data suggest that $ONOO^-$ acts independent of thiol oxidation to cause dilation in isolated

and pressurized PCAs and further support the hypothesis that ONOO⁻-induced dilation is due to nitrosylation and depolymerization of VSM F-actin.

Although the relationship between NT and F-actin in the present study is correlative in nature, there is strong evidence to support a role for nitrosylation in the depolymerization of F-actin (28). ONOO⁻ has been shown to cause depolymerization of F-actin and inhibit polymerization in vitro (4, 16, 51). In addition, studies in neutrophils and red blood cells showed a loss of F-actin and actin-dependent functions following exposure to ONOO⁻ that was associated with increased NT (16, 22). Other nitrogen donors have been shown to reduce F-actin through nitrosylation (3, 18). Finally, a similar study in lung epithelium showed that ONOO⁻-induced NT colocalized with F-actin and resulted in actin depolymerization (28). We therefore hypothesize that nitrosylation is a mechanism causing disruption of the normal state of F-actin, resulting in depolymerization, possibly through structural/conformational changes to actin (44, 52).

However, it is important to note that the present study does not show a specific cause and effect of nitrosylation of actin by ONOO⁻ leading to depolymerization. It is therefore unclear whether the loss of F-actin is due specifically to ONOO⁻-induced NT. Other oxidants such as superoxide and hydrogen peroxide have been shown to alter the actin cytoskeleton. Hydrogen peroxide has been shown to oxidize protein residues of G-actin and induce protein cross-linking, preventing polymerization (6, 20, 41), whereas superoxide in the cerebral circulation activates second messenger systems such as Rac and heat shock proteins, resulting in polymerization of F-actin (19, 42). It is possible that nitrogen donation by ONOO⁻ may not only nitrosylate tyrosine but other protein residues as well, which are not marked by the NT antibody. Additionally, ONOO⁻ may act to modulate second messenger systems, much like superoxide, resulting in reduced F-actin, although currently there are no studies that support this hypothesis.

Similar to NT, the relationship between the loss of F-actin and dilation of tone was also correlative in this study. However, numerous studies have shown that a decrease in F-actin results in decreased contraction and contractile properties in smooth muscle (24, 49, 57, 58). For example, exposure of PCAs to cytochalasins, compounds that cause depolymerization of actin filaments, results in dilation with significant loss of F-actin (48). F-actin is an integral component of the contractile apparatus, and therefore depolymerization would be expected to interfere with the contractility of VSM (14). In addition, since a dynamic actin cytoskeleton is necessary for proper myogenic activity in VSM (13–15), this is a likely mechanism of VSM relaxation and dilation of arteries due to decreased F-actin.

We utilized confocal microscopy to show changes in NT and F-actin in VSM that allowed for analysis of the association between NT, F-actin, and diameter of pressurized vessels. In addition, confocal microscopy also allowed for the determination of colocalization of NT and F-actin in the same vessels. Although other groups have shown that NT can affect the polymerization state of actin in vitro (16) with biochemical assays, those methods require greater amounts of tissue than is possible in these studies. Since nonpressurized cerebral resistance arteries have reduced F-actin and are unable to produce spontaneous tone, pressurized vessels are necessary in order to study myogenic activity. Therefore we investigated the role of

ONOO⁻ in regard to nitrosylation of F-actin in pressurized PCAs with confocal microscopy.

It is unclear whether dilation and loss of F-actin due to ONOO⁻ are reversible. Washing out ONOO⁻ within the 1-h experimental time course had no effect on diameter (unpublished data), suggesting that the ONOO⁻-induced dilation is irreversible. Whether this is due to apoptosis or cell damage not related to apoptosis is also unclear. We have hypothesized that nitrosylation interferes with the normal state of F-actin resulting in depolymerization, possibly through structural changes to actin (44, 52). In support of this, others have shown that nitrosylation can be a form of posttranslational protein modification resulting in targeting and accelerated degradation of proteins by activated peroxisomes (50). This degradative process may be acting to cleave and degrade actin filaments after exposure to ONOO⁻. Whether nitrosylation is reversible or actin protein turnover must occur for functional recovery has yet to be elucidated. However, the possible existence of “protein nitrases” suggests that an endogenous mechanism to denitrify modified residues may exist (27, 34). This may present a possible therapeutic target for interfering with the damaging affect of ONOO⁻-induced nitrosylation and VSM dysfunction.

In summary, exposure of isolated and pressurized PCAs to ONOO⁻ resulted in a loss of myogenic tone and diminished F-actin, in agreement with our previous findings (39). The present study further showed that ONOO⁻ exposure resulted in a significant increase in NT content in VSM. In addition, the loss of F-actin was significantly inversely correlated with the change in both diameter and NT content. Furthermore, F-actin and NT were colocalized in VSM as analyzed by confocal microscopy. The fact that F-actin and NT are both inversely correlated and colocalized suggests a possible causality to dilation. Taken together, these data support our hypothesis that nitrosylation of F-actin may be one mechanism by which ONOO⁻ results in diminished F-actin in the VSM of PCAs, leading to a loss of myogenic tone and reactivity to pressure the consequences of which could further brain injury by decreasing cerebrovascular resistance and increasing edema formation after ischemia and reperfusion injury as well as other disease states such as traumatic brain injury.

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