nature medicine

Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery

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Here we show that ischemia induces sustained contraction of pericytes on microvessels in the intact mouse brain. Pericytes remain contracted despite successful reopening of the middle cerebral artery after 2 h of ischemia. Pericyte contraction causes capillary constriction and obstructs erythrocyte flow. Suppression of oxidative-nitrative stress relieves pericyte contraction, reduces erythrocyte entrapment and restores microvascular patency; hence, tissue survival improves. In contrast, peroxynitrite application causes pericyte contraction. We also show that the microvessel wall is the major source of oxygen and nitrogen radicals causing ischemia and reperfusion–induced microvascular dysfunction. These findings point to a major but previously not recognized pathophysiological mechanism; ischemia and reperfusion-induced injury to pericytes may impair microcirculatory reflow and negatively affect survival by limiting substrate and drug delivery to tissue already under metabolic stress, despite recanalization of an occluded artery. Agents that can restore pericyte dysfunction and microvascular patency may increase the success of thrombolytic and neuroprotective treatments.

Despite substantial progress in understanding the mechanisms of ischemic stroke over the past few decades, there is still no effective therapy. Reperfusing brain shortly after ischemia can improve recovery, but if delayed more than a few hours it has no beneficial effect and may be deleterious[1,2]. Consequently, there is a need to understand the mechanisms of reperfusion-induced injury so that tissue survival can be promoted without further damaging the ischemic brain. The neurovascular unit, composed of the endothelium, astrocytes, pericytes, smooth muscle cells, proteins and enzymes in the extracellular matrix, is considered a major target of reperfusion injury[3,4]. Dysfunction of the neurovascular unit may disrupt microcirculation and hence, promote progression of the infarct to penumbra in addition to contributing to hemorrhagic transformation and edema.

It was known for quite some time that clogging of the microcirculation by cellular elements of blood, fibrin and swollen endothelium and astrocyte endfeet, may impede microcirculation despite recanalization of an occluded artery in rodents as well as primates[5–8]. Mechanisms of this impaired reperfusion (better known as the 'no-reflow' phenomenon) after middle cerebral artery (MCA) occlusion differ at various portions of the microvascular bed[9]. Precapillary arterioles generally remain open, but postcapillary venules are crowded with adherent leukocytes or show complex cellular aggregates consisting of fibrin, activated platelets and/or leukocytes. Capillaries contain fibrin-platelet deposits, entrapped erythrocytes or leukocytes. Compression by swollen endothelium and astrocyte endfeet was postulated as the mechanism of segmental narrowing of capillaries observed in ultrastructural studies[10,11].

However, the role of pericytes in acute microcirculatory disturbance after ischemia and reperfusion has not been investigated. Pericytes have a key role in regulation of capillary blood flow by contracting and dilating with various chemical stimuli originating from neighboring astrocytes and neurons[12]. They may be damaged by oxygen and nitrogen radicals that are intensely generated on the microvascular wall during ischemia and reperfusion[13–15]. Here we show that pericytes on microvessels contract during ischemia and remain contracted despite reopening of the occluded MCA. Erythrocytes are trapped at the capillary constrictions caused by pericyte contractions and obstruct microcirculation. We also show that peroxynitrite causes pericyte contraction, whereas suppressing oxidative-nitrative stress alleviates ischemia and reperfusion-induced pericyte contraction and, thus, positively affects tissue survival.

RESULTS

Pericytes contract and impede microcirculation after ischemia

Microvessels appeared as continuous, thread-like structures when we visualized microvessel lumens by means of horseradish peroxidase (HRP) (**Fig. 1a**). In contrast, HRP-filled ischemic microvessels were often interrupted by node-like constrictions (**Fig. 1a**). After 2 h of ischemia and 6 h of reperfusion ($n = 9$ mice), 48% ± 14% of microvessels ($n = 701$) showed constrictions, whereas only 15% ± 13% ($n = 747$) and 15% ± 9% ($n = 678$) showed discontinuities in nonischemic contralateral hemispheres and naive brains ($n = 2$), respectively. Because we did not perfuse brains to flush blood cells before decapitation, erythrocyte columns filling the nonischemic

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Received 17 March; accepted 29 July; published online 30 August 2009; doi:10.1038/nm.2022

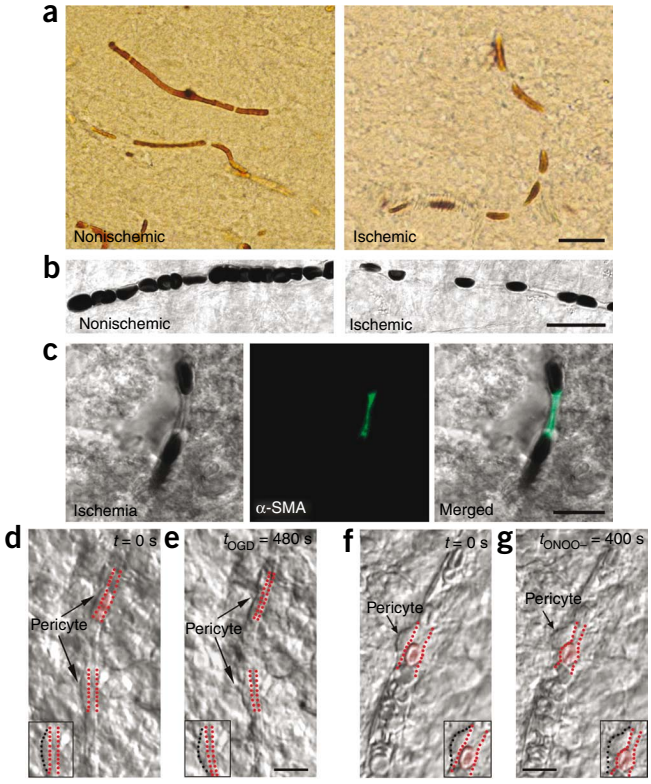

Figure 1 Ischemia and reperfusion induces segmental narrowing of capillaries due to sustained contraction of pericytes. (**a**–**c**) Capillary lumens were visualized by means of bright-field (**a**), DIC (**b**) and DIC and confocal imaging (**c**). In nonischemic hemispheres, HRP-filled microvessels appeared as slender, thread-like structures. In contrast, node-like discontinuities were noted along the course of microvessels in the ischemic hemisphere (**a**). The DIC images illustrate frequent interruptions in the erythrocyte column in an ischemic capillary contrary to a continuous row of erythrocytes flowing through an intact capillary (**b**). Nodal constrictions on the ischemic capillaries colocalized with α-SMA–positive pericytes (green, **c**). Images were captured from the frontal cortex 6 h after 2 h of ischemia. (**d**–**g**) Pericytes visualized live in the isolated mouse retina *in vitro*. Pericytes were identified by their characteristic 'bump on a log' location on the abluminal wall of microvessels (arrows). Representative frames from video recordings illustrate pericytes before (**d**) and after (**e**) OGD. OGD induced pericyte contraction and constriction of the capillary lumen under the pericyte (red dots). Peroxynitrite application also caused pericyte contraction (compare counters of the pericyte soma marked with black dots in insets) and capillary constriction (**f**,**g**). Numbers at the right upper corner indicate the time elapsed after OGD or peroxynitrite ($ONOO^-$) application. Scale bars, 20 μm (**a**,**b**); 10 μm (**c**–**g**).

microvessels were clearly visible with differential interference contrast (DIC) microscopy (**Fig. 1**). In contrast, erythrocyte columns were broken between the constricted segments within ischemic capillaries (**Fig. 1b**). We thought that pericytes might have caused these nodal constrictions, because they have contractile capability and are intermittently spaced on capillaries unlike astrocyte endfeet that diffusely cover microvessels. Indeed, when we labeled pericytes with an antibody against α-smooth muscle actin (SMA), we found that the constricted microvessel segments colocalized with pericytes (**Fig. 1c**). Consecutive optical sections taken every 0.8 μm showed that pericyte cytoplasm decorated with α-SMA filaments ensheathed and constricted the microvessels at these sites (**Fig. 2**).

To directly observe pericyte contraction induced by ischemic-like conditions, we examined isolated retinas live *in vitro*. Of the 25 pericytes monitored, nine contracted within 420 ± 42 s after switching to oxygen-glucose deprived (OGD) solution, which caused a decrease in luminal diameter of adjoining capillary from 5.6 ± 0.8 μm to 3.6 ± 0.7 μm (38%, $P < 0.05$, **Fig. 1d**,**e**). Resumption of perfusion with the physiologic solution did not restore pericyte contraction and capillary constriction (39% ± 4% and 40% ± 9% of the initial diameter at the end of OGD and 30 min reoxygenation, respectively, $n = 3$).

We assessed ischemia and reperfusion–induced pericyte contraction *in vivo* on α-SMA–immunostained brain sections, where pericytes are normally visualized as round-ovoid cells encircling capillary lumens (**Fig. 3**). In contrast, we noticed intense, contracted α-SMA immunostaining and capillary constriction on microvessel cross-sections in ischemic hemispheres (**Fig. 3e**–**h**). We did not observe the ischemia-induced condensation in α-SMA immunopositivity with NG2 immunostaining; hence, we preferred to use α-SMA in this study (**Fig. 2**). When we compared the homolog frontoparietal regions, the mean diameter of pericyte-encircled lumens was significantly lower in hemispheres subjected to ischemia and reperfusion (6.7 ± 0.1 μm versus 10.4 ± 0.2 μm; $n = 150$ vessels, three mice for each group)

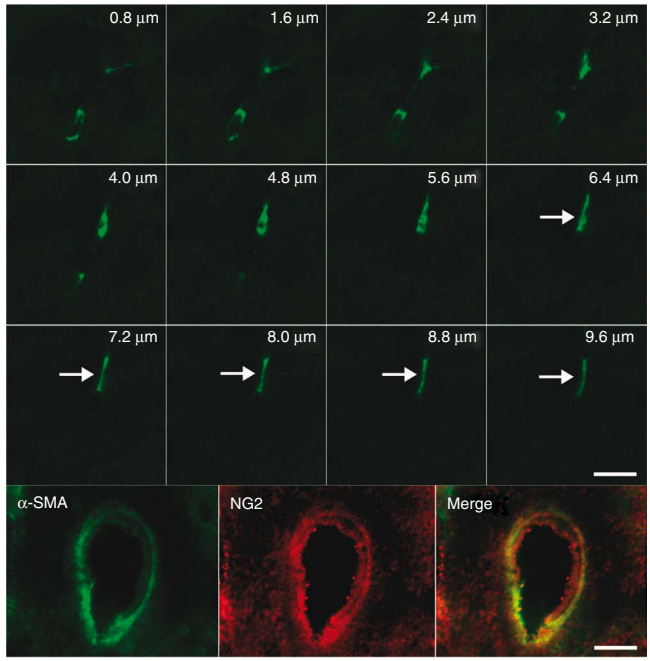

Figure 2 Ischemic pericytes contract and hence constrict the adjoining capillary. Successive confocal sections captured at 0.8-μm intervals of the capillary in **Figure 1c**, showing that it was ensheathed and segmentally constricted by pericyte cytoplasm decorated with α-SMA filaments (green). The contracted pericyte segment is approximately 3.2 μm in thickness (arrows in sections from 6.4 to 9.6 μm) and shows condensed strip-like immunolabeling, unlike the heterogenous and diffuse staining in the other stacks above this level that illustrate the rest of the pericyte(s) cytoplasm surrounding the unconstricted part of the capillary. The single optical section in **Figure 1c** corresponds to the image taken at 8 μm in the composite image illustrated here. Scale bar, 10 μm. Bottom row, microvascular cells stained positively with an antibody against α-SMA (green) were also positively stained with antibody to NG2 (another pericyte marker[43]) (red). They partially overlap in the same cell (yellow in the merged image) because NG2 is mainly localized on membranes, whereas α-SMA is confined to cytoplasm. NG2 also stained the proteoglycan-rich extracellular matrix; hence, the microvessel images were less delineated from the background compared to the contrast observed with α-SMA staining. Scale bar, 5 μm.

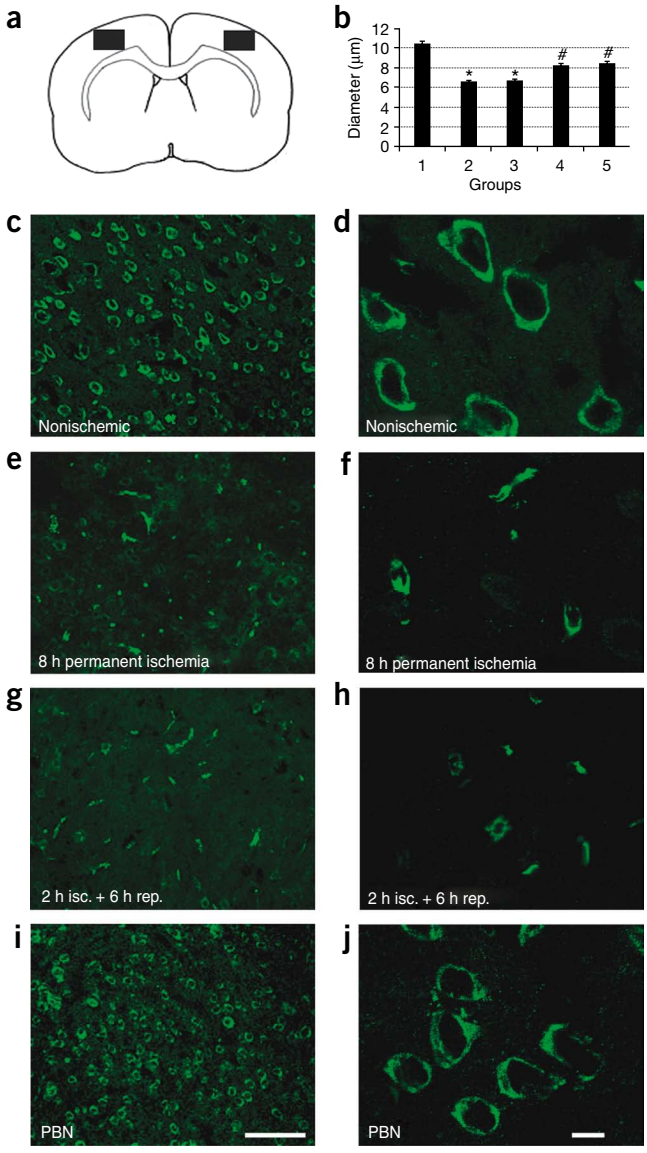

Figure 3 Suppression of oxidative-nitrative stress during reperfusion relieves pericyte contraction and restores microvascular patency. (**a**) Black rectangles in the homolog frontoparietal regions illustrate the areas where microvessel patency was evaluated by measuring diameters of the pericyte-encircled lumens. (**b**) Graph depicts the mean diameter of pericyte-encircled lumens in nonischemic hemispheres (1) and in ischemic hemispheres obtained from mice subjected to 8 h of permanent MCA occlusion (2), to 2 h of ischemia and 6 h of reperfusion (3) and to 2 h of ischemia and 6 h of reperfusion plus L-NA (4) or PBN (5) treatment given just before reperfusion. Error bars represent s.e.m. *, significantly different compared to nonischemic hemisphere (1); #, significantly different compared to nonischemic hemiphere (1) as well as to untreated ischemic groups (2 and 3) $P < 0.05$. (**c**–**j**) Low-power (**c**,**e**,**g**,**i**) and high-power (**d**,**f**,**h**,**j**) confocal images acquired from the cortex marked with the black rectangles in **a**. (**c**,**d**) Nonischemic hemisphere; (**e**,**f**) 8 h of permanent ischemia; (**g**,**h**) 6 h after 2-h transient ischemia; (**i**,**j**) 6 h after 2-h transient ischemia plus treatment with PBN at reperfusion. Pericytes identified with α-SMA immunostaining appear as round-ovoid cells encircling the lumen in the nonischemic hemisphere. In ischemic hemispheres, constricted capillary lumens are surrounded by condensed and contracted α-SMA immunostaining, both of which are restored with PBN treatment. Scale bars: 100 μm (**c**,**e**,**g**,**i**) or 10 μm (**d**,**f**,**h**,**j**).

(**Fig. 3b**). Pericytes contracted to a similar degree to that observed after transient ischemia at the end of 8 h of permanent MCA occlusion (6.6 ± 0.1 μm, n = 150 vessels, three mice), indicating that 6 h of reperfusion after 2 h of ischemia is unable to relieve the ischemia-induced pericyte contraction.

Oxidative-nitrative stress induces pericyte contraction

Ischemia and reperfusion induces intense 3-nitrotyrosine immunolabeling on capillaries (**Fig. 4**). To study the potential role of oxygen and nitrogen radicals in ischemia and reperfusion–induced pericyte contraction, we treated mice with the superoxide scavenger *N*-tert-butyL-α-phenylnitrone (PBN, 100 mg per kg body weight) and a low dose of NOS inhibitor *N*ω-nitro-L-arginine (L-NA, 1 mg per kg body weight) just before reperfusion. Both agents significantly restored microvessel patency measured on cross sections of α-SMA–labeled capillaries (8.4 ± 0.2 μm and 8.2 ± 0.2 μm, respectively) compared to the untreated mice (6.7 ± 0.1 μm; n = 150 vessels, three mice for each group) (**Fig. 3b**,**i**,**j**). Similarly, the percentage of HRP-filled microvessels showing node-like constrictions decreased to 14% ± 11% (n = 761 vessels, three mice) and 23% ± 11% (n = 628 vessels, three mice) with PBN and L-NA treatments, respectively (data not shown). The latter values were not significantly different than values observed in nonischemic hemispheres. We have previously shown that treatment with 1 mg per kg body weight of L-NA lowers nitric oxide (NO) surge, leading to less peroxynitrite formation without suppression of basal endothelial NO synthesis[16,17]. Indeed, we found no significant difference in the mean diameter of pericyte-encircled microvessel lumens before and after this low dose of L-NA in nonischemic hemispheres (10.4 ± 0.2 μm and 9.7 ± 0.2 μm; $P > 0.05$, n = 150 vessels, three mice for each group).

To selectively test the role of endothelial NO, we administered the blood-brain barrier (BBB)-impermeable nitric oxide synthase (NOS) inhibitor L-N^5-(1-iminoethyl)-ornithine (L-NIO) to neuronal NOS (nNOS)-knockout mice in whom the main source of NO is the endothelial NOS (eNOS), as the inducible NOS is not induced within the first hours of ischemia and reperfusion[18,19]. Ischemia and reperfusion caused capillary constriction in nNOS knockouts, but this was less severe compared to wild-type mice (36% versus 11%), possibly owing to elimination of the parenchymal source of NO; pericyte-encircled lumen diameters were 8.8 ± 0.1 μm and 7.9 ± 0.1 μm in nonischemic and ischemic hemispheres, respectively ($P < 0.05$, n = 200 vessels, two mice). However, inhibition of the eNOS in nNOS-knockout mice with L-NIO (10 mg per kg body weight) before reperfusion restored microvessel patency (8.6 ± 0.1 vs.8.7 ± 0.1 μm, $P > 0.05$, n = 200 vessels, two mice). This low dose of L-NIO, which we selected on the basis of preliminary dose-escalation experiments, does not increase arterial pressure more than 10 mm Hg, because it partially inhibits eNOS (data not shown). Accordingly, it decreases NO surge, causing less peroxynitrite formation without substantially suppressing basal endothelial NO synthesis.

In line with the above findings, microinjection of peroxynitrite into the frontal cortex induced pericyte contraction and capillary constriction (n = 6). The contracted pericytes showed intense 3-nitrotyrosine immunolabeling, whereas we did not observe any pericyte contraction and 3-nitrotyrosine immunostaining in brains injected with an equal volume of decomposed peroxynitrite (n = 3) (**Fig. 4**). Similarly, *in vitro* bath application of peroxynitrite (30 μM) to isolated retina caused pericyte contraction and decrease in luminal diameter of the adjoining capillary from 6.3 ± 0.9 μm to 4.4 ± 0.7 μm (n = 5, $P < 0.05$, **Fig. 1f**,**g**). In parallel to *in vivo* findings, addition of PBN

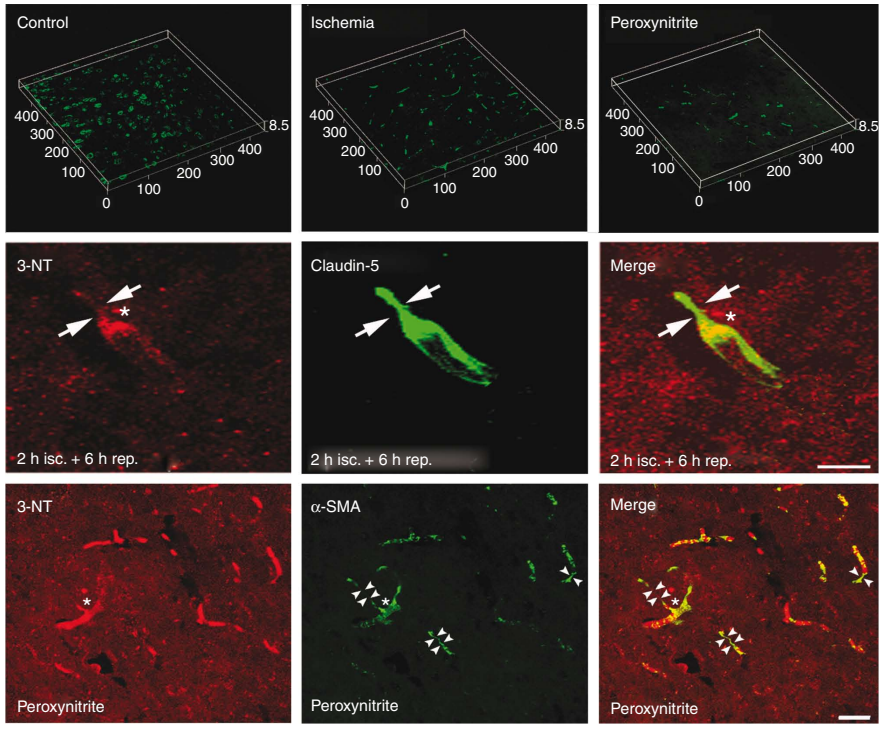

Figure 4 Ischemia- or peroxynitrite-induced pericyte contraction colocalize with 3-nitrotyrosine immunolabeling. Top, three-dimensional reconstruction of *z*-axis images of frontal cortical slabs (465 μm × 465 μm × 8.5 μm) obtained from nonischemic (left) and ischemic (center) hemispheres, illustrating pericytes identified with α-SMA immunolabeling. Unlike diffuse staining of nonischemic pericytes surrounding microvessel lumen, α-SMA immunostaining in ischemic pericytes was contracted and condensed. The image was captured 6 h after 2 h of MCA occlusion. Microinjection of peroxynitrite (right) into the frontal cortex induced contraction of pericytes and condensation of α-SMA similar to ischemia. Middle, left panel shows 3-nitrotyrosine (3-NT, red) immmunolabeling on a capillary after 2 h of ischemia and 6 h of reperfusion. Immunolabeling with another vascular marker, claudin-5 (green), illustrates that nodal constrictions are not an artifact produced by altered α-SMA immunoreactivity. Arrows point to a severely constricted segment and its colocalization (merged images) with intense 3-NT labeling on the capillary wall (*). Scale bar, 5 μm. Bottom, after intracortical peroxynitrite injection microvessel walls were strongly labeled for 3-NT (red). Pericyte cell bodies and processes identified with α-SMA immunoreactivity (green) were also 3-NT immunopositive (yellow color in the merged image). Images illustrate that a pericyte body at the origin of a capillary branch (*) is densely immunostained for 3-NT, and the capillary adjacent to this point is constricted. Constricted microvessel segments are marked with arrowheads. α-SMA staining was condensed in constricted pericytes. Scale bar, 20 μm.

(100 μM)[20] to reoxygenation perfusate relieved pericyte contraction and reduced capillary constriction from 2.5 ± 0.1 μm to 3.5 ± 0.2 μm ($P < 0.05$ and $P > 0.05$, respectively, compared to the initial diameter of 4.1 ± 0.3 μm; $n = 5$) within 510 ± 75 s in contrast to reoxygenation without PBN.

Restoring microvascular patency provides neuroprotection

We assessed the size of ischemic histological damage and neurological outcome 6 h after reperfusion in the treated mice. The ischemic volume was 62 ± 6 mm^3 ($n = 8$) in the saline-treated group, whereas it was significantly lower in L-NIO– (26 ± 7 mm^3, $n = 6$), PBN- (32 ± 5 mm^3, $n = 6$) or L-NA– (45 ± 5 mm^3, $n = 6$) treated groups (**Supplementary Methods** and **Supplementary Fig. 1**). Notably, L-NIO was equally neuroprotective compared to the other two agents that cross the BBB. Neurological examination scores paralleled the size of the ischemic volume (2.6 ± 0.2 arbitrary units (AU) in control, 1.7 ± 0.2 AU in L-NIO–, 1.7 ± 0.2 AU in PBN– and 1.8 ± 0.2 AU in L-NA–treated groups, $P < 0.05$). There were no significant differences between groups regarding the arterial pressure and regional cerebral blood flow recorded during ischemia and reperfusion (**Supplementary Fig. 2**). We also verified that PBN, L-NA or L-NIO treatments inhibited peroxynitrite formation by measuring 3-nitrotyrosine levels in the brain after ischemia and reperfusion (**Supplementary Data** and **Supplementary Fig. 3**).

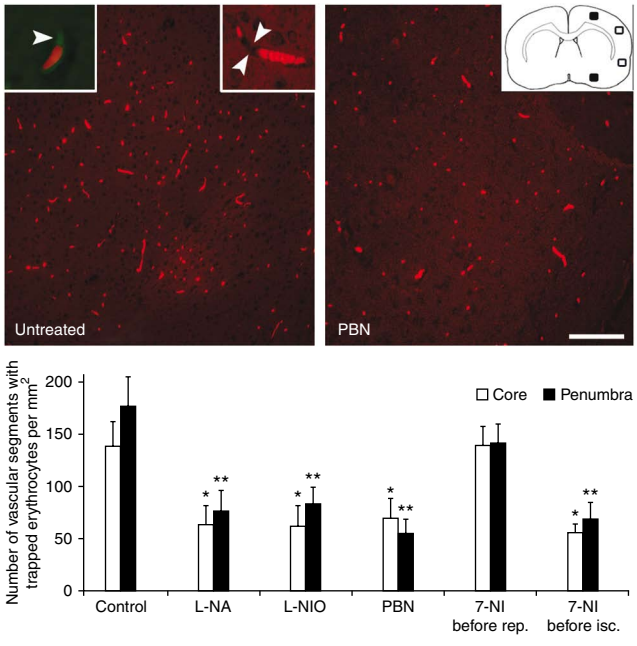

Figure 5 Despite successful recirculation, capillaries in the MCA territory were filled with trapped erythrocytes. Top, fluorescent images illustrate that NaBH4 treatment renders hemoglobulin fluorescent (red) and that there are numerous trapped erythrocytes in capillaries after 2 h ischemia and 6 h reperfusion (left, frontal cortex). Insets illustrate entrapped erythrocyte rouleaus in capillaries around nodal constrictions (arrowheads), colocalizing with contracted pericytes (dense, green α-SMA immunofluorescence in the left inset). PBN treatment markedly lowered the number of capillaries containing trapped erythrocytes (right, frontal cortex). The penumbral (■) and core (□) areas where erythrocyte columns were counted are shown in the inset on the right panel. Scale bar, 100 μm. Bottom, quantification of the number of microvessel segments with trapped erythrocytes per mm^2 in the core and penumbral regions in untreated and L-NA–, L-NIO–, PBN- or 7-NI–treated mice. All agents were administered just before reperfusion. In addition, 7-NI was given before ischemia to another group of mice. Values are given as means ± s.e.m. * and ** denote statistical significance compared to the corresponding control core and penumbral values, respectively ($P < 0.05$).

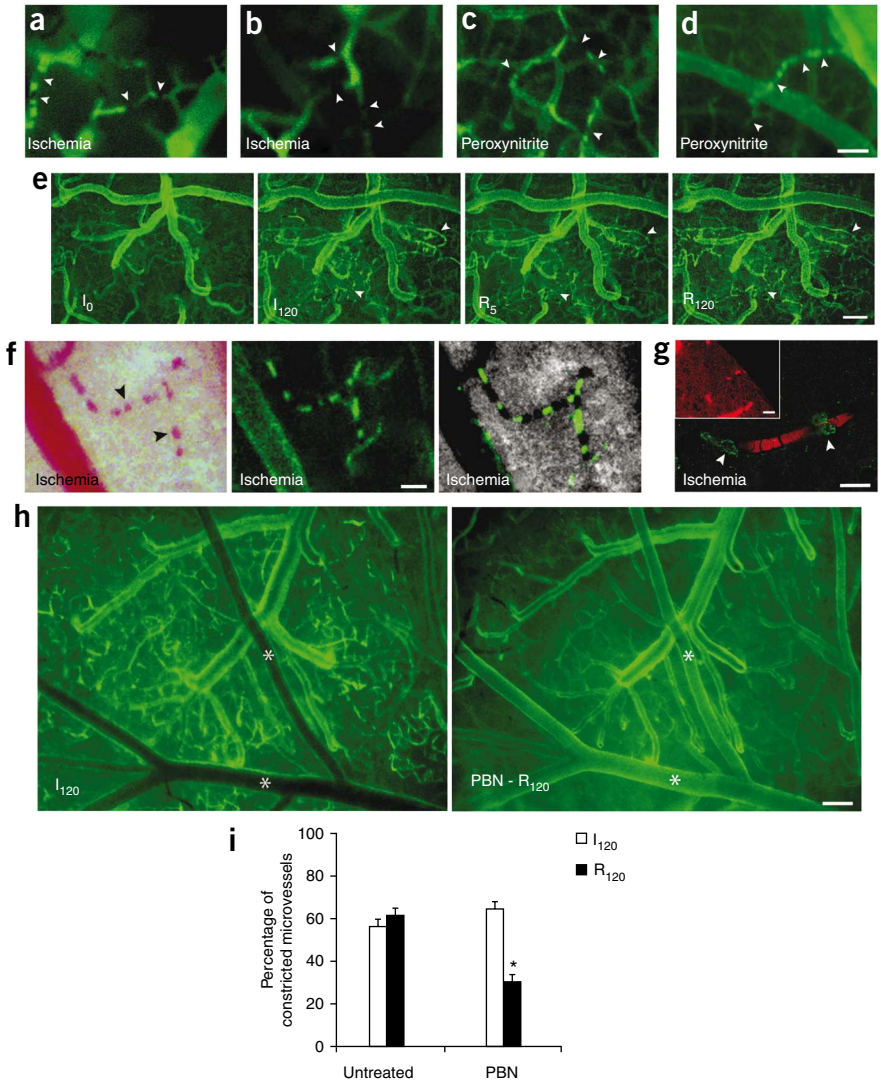

Figure 6 Effects of ischemia and peroxynitrite on microvessels visualized with FITC-dextran and monitored through a cranial window. (**a**–**d**) Numerous capillary constrictions appeared (arrowheads) after MCA occlusion (**a**,**b**) or peroxynitrite application (**c**,**d**). Panel **e** illustrates that capillary segments neighboring constrictions show enhanced FITC-dextran fluorescence due to congestion (arrowheads) and that capillary consrictions and microcirculatory stasis increase during ischemia and do not recover within 2 h after reperfusion. The time (min) that the image was captured during ischemia (for example, I_{120}) or reperfusion (for example, R_{120}) is indicated on lower left corners. (**f**) After ischemia, erythrocytes, which are hardly detectable under bright-field microscopy, were entrapped and became visible around the capillary constrictions (left, arrowheads). Superimposition of bright-field (red erythrocyte columns are pseudo-colored as black) and fluorescent images illustrates that the patent segments between trapped erythrocytes displayed enhanced green fluorescence (FITC-dextran) due to slowed plasma flow. (**g**) Processing of brain sections *ex vivo* with $NaBH_4$ confirmed that constricted capillaries imaged during intravital microscopy are filled with trapped erythrocytes (red, inset) and the constricted parts correspond to SMA-immunolabeled pericytes (green, arrowheads). (**h**) Unlike the constricted and congested capillaries seen before PBN administration (left), most of the microvessels look normal 2 h after PBN administration and reperfusion (right). Asterisks indicate the MCA. **i** depicts the ratio of the constricted capillaries 2 h after ischemia (I_{120}) and reperfusion (R_{120}) in untreated and PBN-treated mice. Values are given as means ± s.e.m. Scale bars: 50 μm (**a**–**d**), 30 μm (**f**), 10 μm (**g** and inset) and 100 μm (**e**,**h**).

Pericyte contraction and consequent microvessel constriction can cause erythrocytes to be trapped within capillaries. Therefore, the number of erythrocyte columns in the MCA territory could be a measure of microcirculatory no-reflow after recirculation. The strong reducing agent $NaBH_4$ renders hemoglobin fluorescent and hence allows for visualization of erythrocytes under fluorescence microscope[21]. Unlike in the HRP experiments, we flushed blood cells with heparinized saline perfusion before killing; hence, capillaries were indistinguishable from background in nonischemic hemispheres, except for the occasional erythrocyte (data not shown). In contrast, there were numerous erythrocyte rouleaus in the core (139 ± 23 per mm^2) and penumbral ischemic regions (178 ± 27 per mm^2) in the saline-treated group 6 h after reperfusion ($n = 8$) (**Fig. 5**). The number of rouleaus at the end of 2 h of ischemia (174 ± 4 per mm^2 and 142 ± 20 per mm^2, in the core and penumbra, respectively, $n = 2$) or after 24 h of reperfusion (155 ± 4 per mm^2 and 176 ± 14 per mm^2, respectively, $n = 2$) was not significantly different, suggesting that pericyte contraction was induced during ischemia and remained so despite recirculation. Entrapped erythrocytes were seen in microvessels of <7 μm diameter (**Fig. 5**). Suppression of the oxidative-nitrative stress significantly lowered the number of microvessels containing trapped erythrocytes in mice treated with L-NIO, PBN or L-NA ($n = 6$ for each group, **Fig. 5**). However, the *in vivo* selective nNOS inhibitor 7-nitroindazole (7-NI, 50 mg per kg body weight) was ineffective when given 30 min before reperfusion ($n = 3$), although it reduced erythrocyte entrapment when administered before ischemia ($n = 3$) (**Fig. 5**).

Supplementary Data and **Supplementary Figure 1** show additional evidence indicating that penumbral survival depends on restitution of capillary flow.

In situ experiments

Finally, we used intravital fluorescent microscopy to observe the effect of ischemia or peroxynitrite on microvessels *in situ* (n = 14 mice). After occlusion of the MCA, numerous constrictions appeared on capillaries. Constricted capillaries showed intense fluorescence, possibly owing to slower passage of FITC–dextran-70S through the contracted segments (**Fig. 6**). We detected entrapped erythrocytes around the constrictions by bright-field microscopy (**Fig. 6f**). Processing of the brain sections with $NaBH_4$ after intravital microscopy confirmed that constricted capillaries in the area imaged were filled with trapped erythrocytes, and the constricted points corresponded to pericytes (**Fig. 6g**). The capillary constrictions and microcirculatory stasis did not noticeably change during the 2-h follow-up after reperfusion; 56% ± 3% and 62% ± 3% of capillaries showed constrictions during ischemia and reperfusion, respectively ($n = 3$ mice) (**Fig. 6e**,**h**). In contrast, PBN given just before reperfusion significantly decreased the ratio of capillaries displaying constrictions to 30% ± 4% ($n = 3$) (**Fig. 6h**,**i**).

We topically applied peroxynitrite over the cortex after adjusting its pH to 7.4. Peroxynitrite induced a modest increase in arteriolar diameter from 27 ± 1 μm to 33 ± 1 μm as reported previously[22], whereas it caused segmental constrictions on 49% ± 2% of capillaries (n = 4 mice) (**Fig. 6c**,**d**). Ebselen (12 mg per kg body weight), a peroxynitrite scavenger, significantly lowered peroxynitrite-induced capillary constrictions to 30% ± 3% (n = 4 mice), whereas its vehicle (47% ± 3%) had no effect (data not shown).

DISCUSSION

The findings of this study can be summarized as follows. First, pericytes contract during ischemia and remain contracted despite reopening of the occluded artery. Second, contracted pericytes induce segmental narrowing of capillaries, which entraps erythrocytes and impedes microcirculation. Third, this abnormality is mimicked by peroxynitrite and is reversed with suppression of peroxynitrite formation and oxidative and nitrative stress. Fourth, restoration of microcirculatory patency promotes tissue survival. Finally, the microvessel wall is a major source of oxygen and nitrogen radicals involved in microvascular injury. Considering that the ischemic changes were evaluated within the first 6 h after reperfusion (that is, when the tissue survival was dependent on restitution of blood flow) and before development of substantial extracellular edema and inflammation, these findings suggest that ischemia and reperfusion–induced injury to pericytes may be a major mechanism that negatively affects tissue survival by limiting substrate delivery to tissue struggling to recover from metabolic perturbation. Capillary constrictions may hinder passage of blood cells and promote their aggregation together with fibrin while allowing plasma flow. Erythrocyte entrapment together with uninterrupted plasma flow can account for the heterogeneous acidity in the penumbra due to mismatch between oxygen and glucose supply and are likely to be crucial in determining the infarct outcome[23]. A study that refuted the possibility of microvascular failure after reperfusion may have reached this conclusion because the microcirculatory patency was assessed with Evans blue, which filled all capillaries along with circulating plasma despite cellular obstruction in the lumen[24].

Pericytes are perivascular cells that are surrounded by the basal lamina and have been shown to closely interact with vascular endothelial cells. Pericytes are associated with arterioles, venules and especially capillaries where there is no smooth muscle[25–27]. They contain contractile proteins and have contractile ability similar to smooth muscle cells in larger vessels. Accordingly, they are thought to have a key role in regulating cerebral blood flow in microvasculature by contracting and relaxing. Indeed, they express a number of receptors for vasoactive mediators such as catecholamines, angiotensin-II, vasoactive intestinal peptide, endothelin-1 and vasopressin. They also modulate the growth and development of endothelial cells, play a part in angiogenesis and differentiation of the BBB and take part in immune responses[27], all of which may participate in late tissue-repair events after ischemia and reperfusion injury.

Unfortunately, there is little information about pericyte biology under ischemic conditions. Future studies may provide better insight to the underlying mechanisms but, with the current information from other ischemic cells, we may infer that oxidative-nitrative stress during reperfusion may hinder restoration of ischemia-induced intracellular calcium overload in pericytes by interfering with mitochondria, endoplasmic reticulum and calcium pumps, which may account for the sustained calcium rise and pericyte contraction[12,14,15,27,28]. Ischemia-induced Rho kinase activation may also increase the sensitivity of the contractile apparatus to intracellular Ca^{2+} by enhancing myosin light chain phosphorylation as does it in smooth muscle cells[29].

We observed a similar degree of neuroprotection and inhibition of 3-nitrotyrosine formation with the BBB-impermeable L-NIO, as compared to L-NA and PBN, which strongly suggests that vasculature was a major source of radicals during reperfusion, although this does not undermine potential contributions of reduced parenchymal toxicity by the BBB-permeable agents. We have excluded the possibility that L-NIO may have penetrated into the parenchyma through the injured BBB because the blood pressure increase induced by L-NIO (a measure of its plasma life) lasted half an hour, suggesting that its action was over before substantial opening of the BBB[30]. nNOS-knockout mice do not have marked parenchymal NO production[31]. Therefore, complete restoration of microvessel constriction with L-NIO given at reperfusion also indicates that excess radical surge from the endothelium at reperfusion is a major player in microvascular injury. Supporting this idea, inhibition of parenchymal NO formation at reperfusion with the nNOS inhibitor 7-nitroindazole in wild-type mice was ineffective in preventing erythrocyte entrapment. These observations are in line with our previously published findings showing that microvessels are the major source of intense NO, superoxide and peroxynitrite production after focal ischemia and reperfusion[16,17]; they point to the importance of radical formation in the microvascular compartment, where the relatively larger endothelial surface/luminal radius ratio and the slower removal of NO by hemoglobin (due to slower flow) may yield to higher peroxynitrite concentrations than in larger vessels during NO and superoxide surges. Therefore, high concentrations of peroxynitrite on the capillary wall may induce a sustained increase in intracellular calcium and pericyte contraction, as shown in cultured central nervous system pericytes exposed to high concentrations of hydrogen peroxide[28], whereas lower peroxynitrite concentrations relax smooth muscle cells in arterioles via ATP-sensitive potassium channels as previously suggested[22,32]. Excess peroxynitrite may also induce pericyte contraction by causing mitochondrial dysfunction and intracellular calcium rise or by depolarizing pericytes via inhibition of potassium channels[32,33].

The possibility of an impaired reflow phenomenon in recirculated brains after global ischemia was initially suggested over 35 years ago[34,35]. A similar microvascular impairment was reported following focal ischemia as well[36]. Later, accumulation of leukocytes primarily in venules in regions with low ischemic flow during early reperfusion was noted[8,37]. The possibility of an impaired microcirculation after focal ischemia has also been emphasized on the basis of ultrastructural studies showing swollen astrocyte endfeet and narrowed, clogged capillary lumens by erythrocytes at early hours of ischemia and, later, by leukocytes[10]. Failure of capillary reperfusion after temporary MCA occlusion was also demonstrated with fluorescent-labeled intravascular markers[38]. In fact, inhibition of neutrophil adhesion and clogging of microcirculation was proposed as a potential mechanism of action for PBN in early studies with nitrones[39] and, more recently, for albumin[40]. Here, we have shown a unique mechanism that may cause segmental narrowing in capillaries due to sustained contraction of pericytes. The active contraction of pericytes probably increases the microcirculatory resistance more than compression of the lumen by swollen endfeet, because large volume changes at the periphery of a cylindrical structure cause relatively smaller changes in diameter. In line with our findings, one study has shown that some pericytes constricted capillaries in isolated retina subjected to *in vitro* ischemia[12]. Of note, although we have shown with intravital microscopy as well *ex vivo* histological studies that half of the capillaries are not adequately perfused after reperfusion, a number of studies, including this one, have found normalized regional cerebral blood flow levels after

reperfusion when detected by laser-Doppler flowmetry[16,40], possibly owing to blood flow bypassing through patent capillaries and arteriolo-veneous anastomoses or thoroughfare channels[41,42].

In conclusion, we show a previously unrecognized pathophysiological mechanism: ischemia and reperfusion may impair capillary reflow by inducing pericyte contraction and, hence, negatively affect survival by limiting substrate and drug delivery to tissue after recanalization of an occluded artery. Oxygen and nitrogen radicals formed in the microvasculature have a key role in pericyte dysfunction in addition to their role in BBB disruption. Therefore, pharmacological agents that can restore pericyte dysfunction and microvascular patency may increase the success of not only thrombolytic but also neuroprotective treatments.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by The Turkish Academy of Sciences (T.D. and Y.G.-O.), Hacettepe University Research Fund 0401105001 (T.D.), Scientific and Technical Research Council of Turkey 104S254 (Y.G.-O.), Ankara University Biotechnology Institute 2001K120240 (A.C.) and Brain Research Association (M.Y.). We are grateful to M.A. Moskowitz for his support and comments. Part of this study was presented at the Society For Neuroscience 37th Annual Meeting in San Diego, California, 2007.

AUTHOR CONTRIBUTIONS

M.Y. performed the *in vivo* experiments and histology studies and contributed to the *in vitro* studies, design of the experiments, data analyses and preparation of the figures; Y.G.-O. conducted and performed the intravital microscopy experiments and *in vitro* studies and contributed to the histology studies, design of the experiments and preparation of the figures; A.V. contributed to the intravital microscopy experiments and *in vitro* studies, performed image analyses and contributed to the preparation of the figures; A.C. conducted the confocal and DIC microscopy studies and prepared the figures; K.T. contributed to the *in vivo* experiments and performed the *in vivo* experiments with knockout mice; T.D. designed and supervised the project, contributed to the data analyses and wrote the manuscript.

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ONLINE METHODS

Animals and stroke model. Experiments were approved by Hacettepe University Animal Experiments Local Ethics Committee (2004/40). We subjected 65 Swiss albino (Hacettepe University Animal Facility) and four nNOS-knockout mice (C57-BL6, the Jackson Laboratory) under ketamine (50 mg per kg body weight intraperitoneally (i.p.)) and xylazine (5 mg per kg body weight, i.p.) anesthesia to 2 h proximal MCA occlusion and 6 h reperfusion using a (8/0)-nylon filament as previously described[16]. We maintained body temperature at 37.0 ± 0.1 °C in all experiments. We subjected additional mice to 2 h ($n = 2$) or 8 h ($n = 3$) of permanent ischemia or to 2 h ischemia and 24 h reperfusion ($n = 2$). We administered PBN, L-NA, L-NIO and 7-NI (Sigma) intraperitoneally in 0.1 ml saline, except for 7-NI, which was dissolved in peanut oil.

Evaluation of microvascular patency with horseradish peroxidase. We intravenously administered 200 mg per kg body weight HRP (Sigma, P8375) in 200 µl saline 20 min before decapitating the mice. We visualized HRP with diaminobenzidine on 60-µm–thick cryosections fixed with 4% paraformaldehyde[10]. We examined 4,464 HRP-filled microvessels (<12 µm) in the ischemic core and contralateral homolog regions.

Immunolabeling of pericytes. We used antibodies against α-SMA (mouse, monoclonal, Neomarkers, MS-113-P1) or NG2 chondroitin sulfate proteoglycan (rabbit, polyclonal, Chemicon, MAB5384 (ref. 43). We blocked nonspecific binding with 10% normal goat serum (Serotec) in PBS at 22–24 °C for 10 min. We incubated sections with primary antibodies (1 in 200) at 4 °C overnight and then with secondary antibodies (1 in 200) for 90 min at 22–24 °C. We used Cy2-conjugated goat antibody to mouse IgG and Cy3-conjugated goat antibody to rabbit IgG (Jackson ImmunoResearch) as appropriate.

Detection of trapped erythrocytes. We transcardially perfused mice with 100 ml of heparinized saline (10 IU ml^{-1}) and then with 4% paraformaldehyde solutions to flush blood vessels before decapitation. We kept their brains in paraformaldehyde for 24 h. We deparaffinized 6 µm–thick slices obtained from paraffin-embedded slabs overnight, hydrated them and then rinsed them with PBS. We treated the sections with 0.2% sodium borohydride in PBS for 30 min and then rinsed them in PBS for 10 min[21].

Peroxynitrite injection. We injected peroxynitrite (Upstate Biotechnology, 20-107) or its decomposed form (Upstate Biotechnology, 20-247) 1 mm deep into the frontal cortex via polyethylene tubing (1 µmol 10 μl^{-1} min^{-1}) or applied it topically over cortex (0.05 µmol 10 μl^{-1} min^{-1}) for 10 min. We dissolved both agents in Tris HCl buffer (pH = 7.4). We fixed sections, cryoprotected them and immunostained them for 3-nitrotyrosine (1 in 100, rabbit polyclonal, Molecular Probes, A-21285), claudin-5 (1 in 100, mouse monoclonal, Zymed, 35-2500) or α-SMA (1in 200, mouse monoclonal, Neomarkers MS-113-P1) as described above.

Studies with isolated retina. We rapidly removed retinas from 6-week-old SV-129 mice (Hacettepe Animal Facility) (Swiss mice could not be used, as their hypopigmented retina could not be distinguished from the choroid) and incubated them for 15 min at 33 °C in 2.5 ml Earle's balanced salt solution (Sigma) supplemented with 0.5 mM EDTA (Merck), 20 mM glucose (Sigma), 15 U papain (Sigma) and 2 mM cysteine (Sigma)[44]. We temporarily kept isolated retinas in a solution containing 140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Na-HEPES, 15 mM mannitol and 5 mM D-glucose, with a total osmolarity of 310 milliosmol l^{-1}. We then transferred retinas (vitreal side up) to the perfusion chamber over an inverted microscope and perfused them with a solution (at 33 °C) containing 124 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM D-glucose. We bubbled the solutions with 95% O_2 and 5% CO_2. To simulate ischemia, we replaced 10 mM glucose with 7 mM sucrose and bubbled with 95% N_2 and 5% CO_2. We simulated reperfusion by switching back to the normoxic, normoglycemic solution. We imaged capillaries (<12 µm) every 10–20 s with the relief contrast DIC technique.

Intravital microscopy. We anesthetized mice with isoflurane during surgery and with urethane (750 mg per kg body weight i.p., followed by 500 mg per kg body weight 30 min later) during the experiment. We kept the arterial pressure, tissue oxygen saturation (Surgivet Pulse Oxymeter) and body temperature within physiological limits. We opened a cranial window of 5 mm × 5 mm over the parietotemporal cortex, leaving the dura intact to maintain physiological conditions. We sealed the window with a glass cover after filling it with artificial cerebrospinal fluid at 37 °C (124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM glucose; pH 7.4). We injected FITC–dextran-70S (0.5 mg in 0.1 ml, Sigma) into the ipsilateral carotid to visualize vessels. We then subjected the mice either to 2 h proximal MCA occlusion and 2 h reperfusion with or without PBN or to topically applied peroxynitrite with or without ebselen (Cayman Chemical). We infused ebselen for 60 min starting 30 min before peroxynitrite. We applied DMSO (60%) for 30 min before peroxynitrite to test effect of the vehicle dissolving ebselen.

Statistical analyses. Values are given as means ± s.e.m. We compared physiological variables, lesion volumes, number of microvessels and biochemical measurements with Kruskal-Wallis test followed by Mann-Whitney *U* test. We compared HRP-filled capillaries with one-way analysis of variance and Dunnett *post hoc* analysis. We compared pericyte-encircled luminal diameters with one-way analysis of variance followed by Tukey *post hoc* analysis. We compared luminal diameters of retinal capillaries with Wilcoxon signed-rank test. We compared groups in intravital microscopy experiments by Mann-Whitney *U* test. We considered $P \leq 0.05$ to be significant.

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doi:10.1038/nm.2022