Tumor Necrosis Factor-α Mediates Hemolysis-Induced Vasoconstriction and the Cerebral Vasospasm Evoked by Subarachnoid Hemorrhage

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Abstract—Hypertension can lead to subarachnoid hemorrhage and eventually to cerebral vasospasm. It has been suggested that the latter could be the result of oxidative stress and an inflammatory response evoked by subarachnoid hemorrhage. Because an unavoidable consequence of hemorrhage is lysis of red blood cells, we first tested the hypothesis on carotid arteries that the proinflammatory cytokine tumor necrosis factor-α contributes to vascular oxidative stress evoked by hemolysis. We observed that hemolysis induces a significant increase in tumor necrosis factor-α both in blood and in vascular tissues, where it provokes Rac-1/NADPH oxidase–mediated oxidative stress and vasoconstriction. Furthermore, we extended our observations to cerebral vessels, demonstrating that tumor necrosis factor-α triggered this mechanism on the basilar artery. Finally, in an in vivo model of subarachnoid hemorrhage obtained by the administration of hemolyzed blood in the cisterna magna, we demonstrated, by high-resolution ultrasound analysis, that tumor necrosis factor-α inhibition prevented and resolved acute cerebral vasocostriction. Moreover, tumor necrosis factor-α inhibition rescued the hemolysis-induced brain injury, evaluated with the method of 2,3,5-triphenyltetrazolium chloride and by the histological analysis of pyknotic nuclei. In conclusion, our results demonstrate that tumor necrosis factor-α plays a crucial role in the onset of hemolysis-induced vascular injury and can be used as a novel target of the therapeutic strategy against cerebral vasospasm. (Hypertension. 2009;54:150-156.)

Key Words: cytokines ■ cerebrovascular disease ■ oxidant stress ■ inflammation ■ subarachnoid hemorrhage

Hypertension can lead to the rupture of a cerebral aneurysm, resulting in sustained subarachnoid hemorrhage (SAH) and eventually cerebral vasospasm.1 This clinical condition is often lethal and provokes severe disability in most survivors.2 Pathophysiologically, the presence of blood in the subarachnoid space induces acute vasoconstriction, causing hyperfusion of the surrounding tissues with devastating consequences for patients.3,4

A substantial amount of studies has been conducted in the effort to predict and treat cerebral vasospasm.5,6 Several pharmacological strategies have been proposed to increase cerebral blood flow in arteries susceptible to vasospasm.5–7 However, their therapeutic effects are limited. Their failure could depend on our lack of knowledge about the pathophysiological mechanisms involved in cerebral vasospasm. Clinical and experimental studies have clarified that brain injury after SAH is a biphasic event with an acute ischemic insult at the time of initial bleed, followed by a generalized cerebral vasospasm. This secondary event is related to the severity of acute vasoconstriction.8,9 Therefore, targeting early pathophysiological mechanisms could interfere with the molecular cascade, leading to the perpetuation of cerebral vasospasm after SAH.

When a cerebral aneurysm undergoes a rupture, bleeding and clot formation occur on the brain surface, where several major blood vessels lay. The contact between blood and the extraluminal wall of arteries provokes vascular injury.10 In particular, blood extravasation induces erythrocyte degradation, leading to hemoglobin release, which evokes vascular oxidative damage by activating NADPH oxidase.11 The latter is constituted by several subunits, which need to be assembled to exert full enzymatic activity. Among these subunits, there is a small G protein, Rac-1, that collects the transduction of several intracellular signalings, converging them on NADPH oxidase activation.12,13 So far, there are no data on the possible involvement of Rac-1 signaling in the vascular injury evoked by hemolysis.
On the other hand, free hemoglobin represents a proinflammatory stimulus that promotes the accumulation of oxygen radicals and upregulates the expression of endothelial and leukocyte adhesion molecules, thereby recruiting macrophages and neutrophils to the site of hemorrhage. This inflammatory response has been suggested to play a role in the onset of cerebral vasospasm after SAH. In this study, we tested the hypothesis that overproduction of an inflammatory trigger, eg, tumor necrosis factor-α (TNF-α), contributes to vascular oxidative stress, which sustains ischemic brain injury after SAH. The first aim of the present study was to investigate the mechanisms of hemolysis-induced vasoconstriction on murine carotid vessels, for which the size allows the analysis of intracellular signaling and its handling with genetic probes. Then, we extended our studies to cerebral vessels, eg, the basilar artery, to verify whether the molecular targets identified on carotid vessels could also play a role in the hemolysis-induced cerebral vasoconstriction. Finally, we tested the relevance of our findings in an in vivo murine model of SAH. In this last set of experiments, beyond the hemolysis-induced oxidative stress and vasoconstriction, we focused our attention on this vascular contraction was not affected by Tiron exposure (maximum vasoconstriction: 816±16 versus 802±11 mg; n=10; P value not significant). Altogether, these data indicate that hemolyzed blood is able to induce vasoconstriction through the activation of oxidative stress mechanisms.

**Materials and Methods**

For a detailed description, please see the online data supplement available at http://hyper.ahajournals.org.

**Results**

**Hemolyzed Blood Induces Vasoconstriction Through Oxidative Stress**

To dissect the molecular mechanisms underlying the vasoconstriction that occurs after contact with blood, we first realized experiments on isolated carotid arteries incubated with whole or hemolyzed blood. The addition of hemolyzed but not whole blood induced a significant vasoconstriction (Figure 1A) as compared with whole blood. On the same vessels, hemolyzed blood induced an increased dihydroethidium (DHE) staining (Figure 1B), revealing an enhanced superoxide production. Furthermore, exposure to Tiron significantly blunted vasoconstriction and oxidative stress induced by hemolyzed blood (Figure 1A and 1B). K⁺-evoked vascular contraction was not affected by Tiron exposure (maximum vasoconstriction: 816±16 versus 802±11 mg; n=10; P value not significant). Altogether, these data indicate that hemolyzed blood is able to induce vasoconstriction through the activation of oxidative stress mechanisms.

**Rac-1/NADPH Oxidase Pathway Mediates Hemolysis-Induced Vascular Oxidative Stress**

Because Rac-1 can be involved in the intracellular signaling converging on NADPH oxidase activation and, consequently, in vascular oxidative stress, we focused our attention on this protein. Interestingly, hemolyzed blood caused an increase in Rac-1 activity as compared with whole blood (Figure S1A). Most important, selective inhibition of Rac-1 by a dominant-negative mutant (AdN17) significantly blunted the action of hemolyzed blood on both oxidative stress and vasoconstriction as compared with the vessels treated with an empty adenovirus (Ad0; Figure S1B and S1C). In contrast, K⁺-evoked vasoconstriction was unaffected by AdN17 (maximum vasoconstriction: 770±17 versus 781±11 mg; n=5; P value not significant).

Moreover, mice with a genetic deletion of p47phox, a cytoplasmic subunit of the NADPH oxidase, were resistant to hemolysis-induced oxidative stress and vasoconstriction (Figure S1D and S1E). K⁺-evoked vasoconstriction was comparable between wild-type and knockout mice (maximum vasoconstriction: 800±14 versus 793±13 mg; n=5; P value not significant). These results clearly demonstrate that the Rac-1/NADPH oxidase pathway plays a crucial role in the hemolysis-induced vascular oxidative stress.

**TNF-α Is a Vasoconstrictive Cytokine and Is Crucial for Hemolysis-Induced Vascular Injury**

It is well known that oxidative stress is generated during inflammatory processes. In this study, we demonstrated both by mRNA transcription and protein expression that hemolysis evoked a marked increase in TNF-α both in blood (Figure 2A and 2B) and in vessels (Figure 2C), suggesting that this inflammatory cytokine could play a role in the genesis of oxidative stress and increased vascular tone in vessels exposed to hemolyzed blood. To verify this hypothesis, we analyzed TNF-α effects on isolated vessels.
TNF-α evoked a dose-dependent vasoconstriction (Figure 2D), lower than that evoked by K⁺ (Δ ID: 39±2 versus 65±3; n=5; P<0.01).

Interestingly, TNF-α was also able to activate vascular Rac-1, as shown by the increase in the Rac-1/p21-activated kinase (PAK) complex (Figure 2E). To demonstrate the relevance of Rac-1 in the vasoconstriction induced by TNF-α, we selectively inhibited Rac-1, infecting the vessels with AdN17, either before the administration of TNF-α or after the onset of TNF-α-evoked vasoconstriction. AdN17 inhibited TNF-α-induced vasoconstriction both before (Figure 2F) and after (Δ ID: from 38±7 to 2±1 µM; P<0.01) TNF-α administration, whereas the use of an empty virus had no effect (Δ ID: from 40±6 to 42±7 µM; P value not significant). These results clearly demonstrate that TNF-α is a vasoconstrictor cytokine that realizes its effect through an intracellular signaling pathway involving Rac-1. Most importantly, blockade of TNF-α by a specific antibody blunted the effects of hemolyzed blood on Rac-1 activation, oxidative stress, and vasoconstriction (Figure 3A through 3C). In contrast, the vasoconstriction evoked by K⁺ was unaffected by the TNF-α antibody (maximum vasoconstriction: 784±18 versus 773±16 mg; n=5; P value not significant). These data reveal the crucial role of the TNF-α/Rac-1 pathway in the molecular cascade converging on hemolysis-induced vascular contraction.

TNF-α Mediates the Vasoconstrictive Effect of Hemolyzed Blood on Basilar Artery

To verify whether the molecular target identified on carotid vessels, namely, TNF-α, also plays a role in the hemolysis-induced cerebral vasoconstriction, we performed experiments on basilar arteries. Also, in this experimental setting, we found that hemolyzed blood induced a significant vasoconstriction as compared with whole blood (Figure S2A). Moreover, hemolyzed blood induced an increased DHE fluorescence (Figure S2B), demonstrating enhanced oxidative stress. The administration of Tiron significantly blunted vasoconstriction and oxidative stress induced by hemolyzed blood. Also in cerebral vessels the administration of exogenous TNF-α evoked a dose-dependent vasoconstriction (Figure S2C).

To evaluate the role of NO in the vascular effects evoked by TNF-α and hemolyzed blood, we performed some experiments during the inhibition of NO obtained with N⁴-nitro-L-arginine methyl ester. Our results demonstrate that N⁴-nitro-L-arginine methyl ester exposure increased basal vascular tone (43±2 mg) but did not significantly affect TNF-α (maximum vasoconstriction: 80±3 versus 88±4 mg; n=5; P value not significant) and hemolyzed blood-induced vasoconstriction (maximum vasoconstriction: 95±6 versus 104±5 mg; n=5; P value not significant). Importantly, TNF-α inhibition blunted the effects of hemolyzed blood on vasoconstriction (Figure S2A). K⁺-evoked vascular contrac-
Figure 3. TNF-α mediates Rac-1 activation, oxidative stress, and vasoconstriction induced by hemolyzed blood (HB). A, Rac-1 activity in carotid arteries after the addition of whole blood (WB) or HB alone and in presence of anti–TNF-α antibody (Ab-TNFα). Representative Western blotting and quantification, corrected for total Rac-1 protein (n=6). B, Representative high-power micrographs and quantification of DHE dyeing in carotid arteries treated with WB or HB alone and in the presence of anti–TNF-α antibody (n=6). C, Vascular response in carotid arteries to HB alone and in the presence of anti–TNF-α antibody (n=6). *P<0.01 vs WB, #P<0.01 vs HB alone.

Finally, we evaluated ACA response and cerebral tissue viability after 2 and 5 days from the infusion of hemolyzed blood into cisterna magna in control conditions and during TNF-α inhibition by either intra-arterial or intraperitoneal administration of an anti–TNF-α antibody. Importantly, this late analysis demonstrated that early TNF-α inhibition is also able to rescue the ACA vasoconstriction observed at 2 (data

Inhibition of TNF-α Rescues Cerebral Vasospasm

We evaluated the effects of TNF-α inhibition in a murine model of SAH obtained by injecting hemolyzed blood into the cisterna magna. In this model, we observed a significant lumen narrowing of the basilar artery associated with thickening of the vascular wall and corrugation of the internal elastic lamina, as well as with increased oxidative stress (Figure 4A and 4B). In particular, mice exposed to SAH showed a 50% reduction in cross-sectional area of the basilar artery as compared with control mice. Importantly, inhibition of TNF-α, realized by infusion of a highly selective anti–TNF-α monoclonal antibody, prevented the phenotypic changes observed in the basilar artery after injection of hemolyzed blood (Figure 4A and 4B).

To monitor the onset and development of cerebral vasospasm in real time, changes in vascular diameter of the anterior cerebral artery (ACA) were also examined by high-resolution ultrasound analysis. In the murine model of SAH, an initial decrease in the ID of ACA was observed 30 minutes after exposure to hemolyzed blood (data not shown). Such phenomenon reached its maximal extension (46%) after ≈60 minutes (Figure 5A) and remained evident for the entire observation period (120 minutes). In contrast, in control mice, saline injection into the cisterna magna did not modify the ACA diameter (data not shown). Interestingly, administration of the anti–TNF-α antibody before the infusion of hemolyzed blood impaired the vasoconstriction of ACA (Figure 5B), whereas it did not exert any effect in control mice. Strikingly, the anti–TNF-α antibody was also able to resolve the already established ACA vasoconstriction induced by hemolysis (Figure 5C).

Figure 4. TNF-α inhibition blocks vasoconstriction and oxidative stress induced by hemolyzed blood (HB) in vivo. A, Immunohistochemical analysis and (B) representative high-power micrographs and quantification of DHE fluorescence of basilar artery sections from mice injected in the cisterna magna with saline (control; n=5) or HB pretreated with a nonimmune IgG (SAH; n=5) or with Infliximab (SAH+infliximab; n=6; ×20 magnification; top right squares are at ×100 magnification). *P<0.01 vs whole blood; #P<0.01 vs HB alone.
not shown) and 5 days after the infusion of hemolyzed blood into the brain (Figure S3A). Moreover, brain 2,3,5-triphenyl-tetrazolium chloride staining, carried out 5 days after, showed a lighter positivity for this in brain sections from mice subjected to SAH in contrast with control mice. This marked difference in 2,3,5-triphenyltetrazolium chloride staining demonstrated that mice subjected to SAH have a reduced brain viability, as a consequence of generalized cerebral ischemia. Strikingly, treatment with the anti–TNF-α antibody infliximab restored 2,3,5-triphenyltetrazolium chloride staining in brain sections from mice subjected to SAH (Figure S3B), thus indicating that early TNF-α inhibition is able to dramatically improve the generalized cerebral ischemia and the impaired brain viability induced by infusion of hemolyzed blood into the cisterna magna. Furthermore, brains from SAH mice showed pyknotic nuclei mainly localized in the posterior cerebral cortex, indicating neuronal damage. Interestingly, infliximab pretreatment protected from neurodegeneration (Figure 6).

**Discussion**

In this study, we demonstrated that TNF-α release was crucial for hemolysis-induced cerebral vasospasm in a murine model of SAH. More important, TNF-α inhibition not only prevented cerebral vasospasm, but was also able to resolve vasospasm when it was already established.

After the rupture of a cerebral aneurysm, blood does not remain fixed in the subarachnoid space but squeezes around making contact with the extraluminal wall of the arteries. At the same time, erythrocyte hemolysis and consequent release of oxyhemoglobin occur.\(^\text{10}\) In our hands, injection of hemolyzed blood in the cisterna magna proved that hemolysis represents a decisive step in inducing cerebral vasospasm. This effect can be reproduced easily in isolated cerebral vessels, where exposure to hemolyzed blood evokes a marked vasoconstriction. This methodological approach has allowed us to characterize intermediate vascular phenotypic changes leading to vasoconstriction. In particular, our data demonstrate that the exposure of isolated cerebral vessels to hemolyzed blood induces a strong oxidative stress. This event is crucial for hemolysis-induced vasoconstriction, because the use of antioxidant agents rescues the hemolysis effect on vascular tone. Interestingly, both hypersensitivity to hydroxyl radicals in the basilar artery and a decreased availability of NO in a subarachnoid space have been reported after SAH.\(^\text{18–20}\)

However, our data show that the inhibition of NO synthesis did not significantly modify the vasoconstrictor effect of hemolyzed blood in isolated vessels, indicating that other mechanisms are involved in reactive oxygen species–mediated vasoconstriction. In agreement with our data, it has been

**Figure 5.** TNF-α inhibition can both prevent and resolve the vasospasm induced by hemolyzed blood (HB). A, Echographic analysis of ACA at 0, 60, and 120 minutes after injection of HB in cisterna magna (n=6) in mice pretreated with a nonimmune IgG; arrow indicates ACA. B, Echographic analysis of ACA at 0, 60, and 120 minutes after injection of HB in cisterna magna of mice pretreated with infliximab (n=5). C, Echographic analysis of ACA at 0, 60, and 120 minutes after injection of HB in cisterna magna of mice treated with infliximab after the occurrence of vasospasm (60 minutes after injection of HB; n=5). Representative images and quantification of ID are shown (*P<0.01 vs 0 minutes; #P<0.01 vs 60 minutes).

**Figure 6.** TNF-α inhibition prevents neurodegeneration induced by SAH. A, Representative photomicrographs of hematoxylin-eosin staining of brain sections of nonimmune IgG (SAH; n=3) and infliximab–treated SAH mice (SAH+infliximab; n=4); ×100 magnification. Arrows indicate pyknotic nuclei. B, Quantification of neuronal damage as number of pyknotic nuclei per millimeter squared (#P<0.05 vs SAH).
reported that reactive oxygen species can exert also a direct vasoconstriction through modulation of calcium levels and/or arachidonic acid metabolism. Therefore, because oxidative stress appears to be a good intermediate phenotype toward cerebral vasospasm, we have investigated the molecular mechanisms involved in the generation of vascular oxidative stress stimulated by hemolysis. We have focused our attention on NADPH oxidase, largely expressed in cerebral arteries. On this issue, a biphasic effect of NADPH oxidase–induced reactive oxygen species generation on vascular tone has been observed. In particular, a low amount of reactive oxygen species induces vasorelaxation, whereas high levels have been reported to induce vasoconstriction in cerebral arteries. NADPH oxidase is activated during SAH, thus causing an increase of superoxide formation and impairing self-regulating vasodilation. These previous data fully support our results showing that vessels with genetic ablation of p47phox are protected from hemolysis-evoked oxidative stress, clearly demonstrating an involvement of NADPH oxidase. Interestingly, hemolyzed blood is able to activate Rac-1. This activation is crucial for oxidative stress and vasoconstriction. Thus, Rac-1 intracellular signaling is an important requisite for hemolysis-induced vascular injury. These results are in agreement with previous studies reporting that oxidative stress plays a significant role in the development of acute brain injury and cerebral vasospasm after SAH. Oxidative stress is also a main component of inflammatory processes and is generated as a response to several inflammatory cytokines. Among the latter, TNF-α can be considered a possible candidate for hemolysis-induced vasoconstriction, because it has been reported that this cytokine is augmented in the subarachnoid space after SAH and correlates with brain damage. However, no mechanistic relationships have been reported to date.

Our data demonstrate increased TNF-α levels in hemolyzed blood. This result is supported by previous evidence showing an increased TNF-α release from circulating macrophages exposed to hemoglobin. More important, in this study we demonstrated for the first time that hemolysis is also a stimulus for TNF-α release in cerebral vascular tissue, likely activating Toll-like receptor 4, which has been described to be overexpressed in the basilar artery after SAH. These results strengthen the hypothesis that TNF-α could play a role in the onset of cerebral vasospasm. This theory finds a strong support in our evidence that TNF-α is able to evoke a direct vasoconstrictor effect on isolated cerebral vessels through Rac-1 activation. Such evidence extends previous observations showing that TNF-α impairs endothelium-dependent vasorelaxation. More important, the inhibition of TNF-α is able to counteract the effects of hemolysis on Rac-1 activation, oxidative stress, and vasoconstriction, thus demonstrating that TNF-α is crucial for the abnormal cerebral vascular tone induced by hemolysis.

For this reason, we targeted TNF-α to verify its relevance in an in vivo model of SAH. Strikingly, our data demonstrate that the inhibition of TNF-α rescues the development of early cerebral vasospasm evoked by the injection of hemolyzed blood into the cisterna magna. Our analysis was accomplished not only by histological evaluation of structural changes in the basilar artery, but also by a novel ultrasound imaging technique that provides continuous real-time monitoring of vascular tone of ACA by evaluating its changes in ID. This analysis allows both temporal and spatial characterizations of cerebral vasospasm and can also be proposed to evaluate the effectiveness of novel therapeutic interventions on cerebral vascular tone. With this approach, we showed the onset and the development of the ACA vasospasm induced by hemolyzed blood for the first time. More importantly, we were able to detect the beneficial effect of TNF-α inhibition in the prevention of vasoconstriction. Excitingly, the blockade of TNF-α activity is also able to resolve the early established cerebral vasospasm, thus revealing that the inhibition of this cytokine is important not only for the onset but also for the perpetuation of hemolysis-induced cerebral vasoconstriction. This conclusion is strongly supported by our late analysis, focused on the brain damage accomplished 2 and 5 days after SAH. In fact, the early inhibition of TNF-α is also able to counteract the chronic vasospasm, thus exerting a real protection for brain against ischemia. Therefore, our strategy focusing on the early phase of cerebral vasospasm has allowed us to reveal a novel molecular mechanism that can be useful to more efficiently fight the long-term injury occurring after SAH. Therefore, the investigation of the early phase of cerebral vasoconstriction is not trivial, as depicted by the results of this study and other previous reports showing that late brain injury is strictly related to early molecular events. On the other hand, the fact that even in our study the inhibition of TNF-α leaves a slight residual cerebral hypoperfusion at late phase reveals that other mechanisms can participate in the action of hemolyzed blood on brain injury, as depicted by previous reports.

However, so far, the proposed therapies for cerebral vasospasm have mainly targeted the delayed phase of cerebral vasospasm, and this approach could explain the failure in the treatment of patients with cerebral vasospasm. In fact, oxyhemoglobin appears early after SAH, thus triggering the whole course of events flowing into cerebral vasoconstriction and hypoperfusion. On the other hand, the use of a symptomatic treatment of cerebral vasospasm with vasodilators has several limitations, because the hypotensive effect favors cerebral ischemia, which is further aggravated by impaired autoregulation of cerebral blood flow after the rupture of the intracranial aneurysm.

In conclusion, our results propose the use of TNF-α inhibitors as a novel therapeutic strategy against cerebral vasospasm in humans. This translation is strongly facilitated by the fact that these drugs are already used in clinical practice in the treatment of several inflammatory diseases.

**Perspectives**

In this study, we identified a novel therapeutic target against cerebral vasoconstriction after SAH, a condition associated with elevated blood pressure levels. In particular, we showed that an inflammatory cytokine, TNF-α, mediated the deleterious effects of hemolyzed blood on vessels, both in vitro and in vivo. Neutralization of TNF-α by administration of infliximab, a TNF-α antibody used in clinical practice, was able to prevent and resolve cerebral vasospasm in a murine model.
Thus, future research will be aimed at evaluating the efficacy of this treatment in patients with SAH, which might limit this lethal consequence of hypertension.

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Disclosures
None.

References
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TNFalpha mediates haemolysis-induced vasoconstriction and the cerebral vasospasm evoked by subarachnoid hemorrhage

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Animals
95 male C57BL6 mice were used (10-12 weeks old; Charles River). Additional experiments were performed using age and weight-matched p47phox^+/− (KO, n=5) mice on a C57Bl/6 background (WT, n=5). Mice were anesthetized by i.p. injection of xylazine/ketamine (20mg/kg and 110mg/kg; Phoenix Pharmaceuticals). All experimental procedures were approved by our institutional review committee and were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Blood and cerebral-spinal fluid
Cerebral-spinal fluid (CSF) samples and blood were taken from hydrocephalus normotensive patients for therapeutical reasons and analysis. They were analyzed (cytochemical and cultural analysis) to ensure absence of infections. Blood was haemolyzed by shaking for several minutes and centrifugation for 10 min at 3000 rpm, and left at room temperature. The study was approved by our institutional review committee and the subjects gave informed consent.

Evaluation of vascular reactivity
After anesthesia, mice were decapitated and carotid and basilar arteries were dissected out, cleaned of adhering perivascular tissue, and placed in cold Krebs-Henseleit buffer (mmol/L: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4·7H2O 1.2, KH2PO4 1.2, NaHCO3 25, glucose 5.6). Carotid arteries were mounted on either pressure or wire myograph. Basilar arteries were mounted on wire myograph. Vascular reactivity was tested as previously described (1-2) and vasoconstriction has been expressed as percentage of maximum K^+ (80mmol/L)-evoked force.

Evaluation of Rac-1 activity
Given the great amount of tissue needed to characterize molecular events in our study we used carotid arteries. Rac-1 activity has been evaluated as previously described (1-2).

Evaluation of oxidative stress in vessels
Analysis of superoxide production was assessed by dihydroethidium (DHE) assays as previously described (1-2).

Adenoviral infection of carotid artery
Pressure myograph was used to allow adenoviral infection of carotid arteries, as previously described (1-2). Carotid arteries were infected with 10^9 pfu/mL AdN17, an adenoviral vector containing a dominant negative mutant of Rac-1 and Green Fluorescent Protein (GFP) as a reporter gene, or with a vector containing only GFP (Ad0) as control, as previously described (1-2). This experimental procedure was impossible to perform, in our experimental system, in basilar artery for the small size of the vessel.
**Evaluation of TNFα expression in blood and vessels**

TNFα expression was evaluated by both real-time PCR and Western blotting. Lymphocytes were isolated from whole and haemolyzed blood and total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA (100 ng) from each sample was transcribed into cDNA using the RT-PCR Superscript III kit (Invitrogen). 2 µl (10% of reverse transcription reaction) of each cDNA preparation were subsequently used as template for PCR containing 1 µmol/L of TNFα primers and 7.875 µl of SYBR green PCR master mix (Applied Biosystems). Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 minutes; 95°C for 5 minutes; 40 cycles 95°C for 45 seconds; and 58°C for 1 minute. TNFα gene expression levels were determined using the Relative Quantification (ΔΔCt) Study of 7500 System SDS Software (Applied Biosystems). Efficiencies of the real time primers were previously tested by PCR.

Western blot analysis was performed on proteins obtained before and after blood haemolysis. Protein content was measured using the Bradford method. Aliquots were subjected to SDS-PAGE, transferred onto nitrocellulose membrane and probed with mouse anti-TNFα (Chemicon, International, CA) overnight at 4°C and with anti-mouse secondary antibody (Amersham). To normalize for protein quantity, membranes were incubated with anti β-actin antibody.

TNFα expression was evaluated also in vascular tissue. Briefly, carotid arteries were treated with whole blood, haemolyzed blood and LPS as positive control. The presence of the cytokine was evaluated 40 minutes after treatment by western blot as above described.

**Murine model of SAH**

After anesthesia, mice were placed in a prone position with the head flexed by approximately 30°, and the atlanto-occipital membrane was exposed. A 1.5 cm linear incision was performed on the posterior scalp of the mouse, on the midline about 5mm above and 10mm below a horizontal plane joining the external auditory meatus. The right femoral artery was exposed and cannulated with a polyethylene catheter (PE-10) and 0.04 ml of autologous blood was withdrawn. An equivalent volume of normal saline solution was replaced i.p. after blood removal. Subsequently exposed atlanto-occipital membrane was punctured with a 30-gauge steel needle, and 0.04 ml of cerebrospinal fluid was aspirated percutaneously from the cisterna magna, and then 0.04 ml of blood was slowly injected at rate of 1.2 ml/h for 2min by infusion pumps (Harvard apparatus) in cisterna magna through a PE-10 catheter, placed in a horizontal position and blocked in situ by fibrin glue tissueol (Baxter). Saline solution was injected in control mice. The animals were then tilted with tail up for 10 min in order to diffuse the blood into the subarachnoid space. Then, the catheter was cut just out of the entry point end, which was previously closed with a microclip to avoid the possible escape of fluid during removal of the tube. Finally, the occipital muscles were sutured and the skin was closed.

**2,3,5-Triphenyltetrazolium chloride and hematoxylin-eosin staining for the evaluation of cerebral tissue damage**

2,3,5-triphenyltetrazolium chloride (TTC) staining was used to differentiate viable tissue from that with impaired viability (3). After 5 days from SAH, brains were quickly isolated, placed in cold PBS and sectioned in to serial 1 mm-thick coronal slices. Isolation and slicing of the brain were completed within 10 min after the mouse had been decapitated. The brain slices were transferred in
TTC incubation medium (0.05% w/v in PBS) for 30 min at 37°C. After staining, slices were washed in PBS and photographed; red colour intensity was assessed by a computer assisted image analysis system (Spot, Universal Imaging). Brain cross-sections (10 µm in thickness) were assessed as described above. Classical hematoxylin-eosin staining was performed to evaluate neuronal damage. Cell death quantification was obtained by counting pyknotic cells showing an intense nuclear condensation, and a rounded shape, in 600 µm distant rostro-caudal brain serial sections of the two treated groups (Bregma levels from –2.06 to –3.16). Data are expressed as the number of pyknotic nuclei per mm².

**Evaluation of vascular diameter**
To evaluate the *in vivo* onset and development of vasospasm after the injection of haemolyzed blood, we monitored the anterior cerebral artery (ACA) by ultrasound analysis using a high-resolution imaging system (Vevo 770; VisualSonics) equipped with a 55 MHz transducer (4). 15 minutes after injection, the mice were anesthetized and laid prone on a platform with all legs taped. The head was placed on the left side. To reveal the anterior cerebral artery the probe was oriented to the anterior side of mouse skull, along the axis connecting the eye and the ear. Vessel diameter was recorded using an internal computer assisted measurement analysis system 30, 60, 120 minutes, and 2 and 5 days after injection of haemolyzed blood. Basilar artery diameter was evaluated ex vivo. Two hours after the injection of haemolyzed blood, mice were subjected to intracardiac perfusion-fixation. In particular, they were perfused with 0,1 mol/L PBS, followed by 25 ml of 4% paraformaldehyde at a flow rate of 2,5 ml/min for 10min. The brain was removed, immersed in the same fixative overnight at 4 °C, rinsed, dehydrated and embedded in paraffin. Cross-sections (5 µm in thickness) of the brain were cut (Leica RM2245 microtome), and placed on microscope slides for hematoxylin-eosin staining to evaluate structural changes in basilar artery. Measurement of vessel diameter was performed using a computer assisted image analysis system (Spot, Universal imaging).

**Infusion of anti-TNFα antibody (Infliximab) in mice**
Administration of Infliximab (Schering Plough), or a non-immune IgG as control, was performed in the model of SAH by either intra-arterial approach (3mg/kg) through the cannulated femoral artery before or 60 min after the injection of haemolyzed blood, or by i.p. injection (4.5mg/kg) during surgical procedures.

**Statistical analysis**
Results are shown as mean ± SEM. Data were analyzed by Student’s t-test or 2-way ANOVA followed by Bonferroni post-hoc analysis, as appropriate, using SPSS 14.0 software.
References


Figure S1. Rac-1 and NADPH oxidase mediate vasoconstriction and oxidative stress induced by HB. 

A. Vascular Rac-1 activity after addition of WB and HB. Representative Western blotting and quantitation, corrected for total Rac-1 protein (n=4). 

B. HB-induced DHE fluorescence in carotid arteries infected with an empty vector (Ad0) or a dominant negative mutant of Rac-1 (AdN17). Representative images and quantification (n=5). 

C. Vascular response to addition of HB on carotid rings pre-infected with Ad0 and AdN17 (n=6). 

D and E. DHE fluorescence (representative images and quantification, and vascular response to HB in WT and in p47 phox -/- mice (n=5). (*p<0.02 vs Ad0 or WT).
Figure S2. Haemolyzed blood induces vasoconstriction and oxidative stress in basilar arteries via TNFα. A, Vascular response in basilar arteries to WB (empty bars) or HB (full bars), alone and in presence of Tiron or anti-TNFα antibody (n=6). (*p<0.01 vs whole blood, #p<0.01 vs haemolyzed blood alone). B, Quantification of oxidative stress in basilar arteries, evaluated as DHE fluorescence (n=4). (*p<0.01 vs control; #p<0.01 vs HB). C, Dose-dependent vascular response in basilar arteries to TNFa (n=4, *p<0.01 vs basal).
**Figure S3.** TNFα inhibition prevents chronic effects of vasospasm induced by SAH. A, Echographic analysis of anterior cerebral artery (ACA) at 5 days from SAH, in control mice (n=4), non-immune IgG (SAH, n=5) and infliximab treated SAH mice (SAH+infliximab, n=6). Representative images of internal diameter (ID) are shown. B, A set of seven rostro-caudally serial slices of mouse brains and relative quantification, from the same experimental groups indicated above, incubated with 0.05% TTC in PBS. AU = Arbitrary Units. * p<0.05 vs Control, #p<0.05 vs SAH.