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

Carmine Vecchione, Alessandro Frati, Alba Di Pardo, Giuseppe Cifelli, Daniela Carnevale, Maria Teresa Gentile, Rosa Carangi, Alessandro Landolfi, Pierluigi Carullo, Umberto Bettarini, Giovanna Antenucci, Giada Mascio, Carla Letizia Busceti, Antonella Notte, Angelo Maffei, Gian Paolo Cantore, Giuseppe Lembo

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 vasospasms. 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
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 histological examination, we developed a novel ultrasound imaging analysis, which allows a real-time in vivo evaluation of the onset and progression of cerebral vasospasm.

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 vasconstriction (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 vasconstriction 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 vasconstriction. AdN17 inhibited TNF-α-induced vasconstriction 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 important, blockade of TNF-α by a specific antibody blunted the effects of hemolyzed blood on Rac-1 activation, oxidative stress, and vasconstriction (Figure 3A through 3C). In contrast, the vasconstriction evoked by K⁺ was unaffected by the TNF-α antibody (maximum vasconstriction: 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 vasconstriction, we performed experiments on basilar arteries. Also, in this experimental setting, we found that hemolyzed blood induced a significant vasconstriction 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 vasconstriction and oxidative stress induced by hemolyzed blood. Also in cerebral vessels the administration of exogenous TNF-α evoked a dose-dependent vasconstriction (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 N^G^-nitro-L-arginine methyl ester. Our results demonstrate that N^G^-nitro-L-arginine methyl ester exposure increased basal vascular tone (43±2 mg) but did not significantly affect TNF-α (maximum vasconstriction: 80±3 versus 88±4 mg; n=5; P value not significant) and hemolyzed blood-induced vasconstriction (maximum vasconstriction: 95±6 versus 104±5 mg; n=5; P value not significant). Importantly, TNF-α inhibition blunted the effects of hemolyzed blood on vasconstriction (Figure S2A). K⁺-evoked vascular contrac-
tnation was not affected by Tiron (maximum vasoconstriction: 261±11 versus 256±10 mg; n=10; P value not significant) or TNF-α inhibition (maximum vasoconstriction: 269±8 versus 275±8 mg; n=5; P value not significant). These data indicate that TNF-α is involved in the action of hemolyzed blood on vascular tone also in cerebral arteries.

**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).

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

**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.

**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; x20 magnification; top right squares are at x100 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/H9251 antibody 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. 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.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

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**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).*

![Image of Figure 6](image-url)

**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.\textsuperscript{18,21,22} 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.\textsuperscript{23,24} 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.\textsuperscript{23,24} NADPH oxidase is activated during SAH, thus causing an increase of superoxide formation and impairing self-regulating vasodilation.\textsuperscript{24,25} 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.\textsuperscript{26,27} Oxidative stress is also a main component of inflammatory processes and is generated as a response to several inflammatory cytokines. Among the latter, TNF-\textalpha is 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.\textsuperscript{28} However, no mechanistic relationships have been reported to date.

Our data demonstrate increased TNF-\textalpha levels in hemolyzed blood. This result is supported by previous evidence showing an increased TNF-\textalpha release from circulating macrophages exposed to hemoglobin.\textsuperscript{15} More important, in this study we demonstrated for the first time that hemolysis is also a stimulus for TNF-\textalpha release in cerebral vascular tissue, likely activating Toll-like receptor 4, which has been described to be overexpressed in the basilar artery after SAH.\textsuperscript{29,30} These results strengthen the hypothesis that TNF-\textalpha could play a role in the onset of cerebral vasospasm. This theory finds a strong support in our evidence that TNF-\textalpha is able to evoke a direct vasoconstrictor effect on isolated cerebral vessels through Rac-1 activation. Such evidence extends previous observations showing that TNF-\textalpha impairs endothelium-dependent vasorelaxation.\textsuperscript{31} More important, the inhibition of TNF-\textalpha is able to counteract the effects of hemolysis on Rac-1 activation, oxidative stress, and vasoconstriction, thus demonstrating that TNF-\textalpha is crucial for the abnormal cerebral vascular tone induced by hemolysis.

For this reason, we targeted TNF-\textalpha to verify its relevance in an in vivo model of SAH. Strikingly, our data demonstrate that the inhibition of TNF-\textalpha 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-\textalpha inhibition in the prevention of vasoconstriction. Excitingly, the blockade of TNF-\textalpha 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 vasospasm. 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-\textalpha 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.\textsuperscript{28} On the other hand, the fact that even in our study the inhibition of TNF-\textalpha 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.\textsuperscript{32–34}

However, so far, the proposed therapies for cerebral vasospasm have mainly targeted the delayed phase of cerebral vasospasm,\textsuperscript{35,36} and this approach could explain the failure in the treatment of patients with cerebral vasospasm. In fact, oxyhemoglobin appears early after SAH,\textsuperscript{9} thus triggering the whole course of events flowing into cerebral vasospasm and hypoperfusion. On the other hand, the use of a symptomatic treatment of cerebral vasospasm with vasodilators has several limitations, because the hypertensive effect favors cerebral ischemia,\textsuperscript{37} 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-\textalpha 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.\textsuperscript{38,39}

**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-\textalpha, mediated the deleterious effects of hemolyzed blood on vessels, both in vitro and in vivo. Neutralization of TNF-\textalpha by administration of infliximab, a TNF-\textalpha 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.

Source of Funding
This work was partly supported by the Italian Ministry of Health.

Disclosures
None.

References