Impaired vascular responses of insulin-resistant rats after mild subarachnoid hemorrhage

Adam Institoris,1,2 James A. Snipes,1 Prasad V. Katakam,1 Ferenc Domoki,2 Krisztina Boda,3 Ferenc Bari,3 and David W. Busija1

1Department of Physiology and Pharmacology, Wake Forest University Health Sciences, Winston-Salem, North Carolina; and Departments of 2Physiology and 3Medical Informatics and Medical Physics, School of Medicine, University of Szeged, Szeged, Hungary

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Impaired vascular responses of insulin-resistant rats after mild subarachnoid hemorrhage. Am J Physiol Heart Circ Physiol 300: H2080–H2087, 2011. First published March 18, 2011; doi:10.1152/ajpheart.01169.2010.— Insulin resistance (IR) impairs cerebrovascular responses to several stimuli in Zucker obese (ZO) rats. However, cerebral artery responses after subarachnoid hemorrhage (SAH) have not been described in IR. We hypothesized that IR worsens vascular reactions after a mild SAH. Hemolyzed blood (300 µl) or saline was infused (10 µl/min) into the cisterna magna of 11–13 wk-old ZO (n = 25) and Zucker lean (ZL) rats (n = 25). One day later, dilator responses of the basilar artery (BA) and its side branch (BA-Br) to acetylcholine (ACh, 10–6 M), cromakalim (10–7 M, 10–6 M), and sodium nitroprusside (10–7 M) were recorded with intravital videomicroscopy. The baseline diameter of the BA was increased both in the ZO and ZL rats 24 h after the hemolysate injection. Saline-injected ZO animals showed reduced dilation to ACh (BA = 9 ± 3 vs. 22 ± 4%, and BA-Br = 23 ± 5 vs. 37 ± 7%) compared with ZL rats. Hemolysate injection blunted the response to ACh in both the ZO (BA = 4 ± 2% and BA-Br = 12 ± 3%) and ZL (BA = 7 ± 2%, and BA-Br = 11 ± 3%) rats. Cromakalim (10–6 M)-induced dilation was significantly reduced in the hemolysate-injected ZO animals compared with the saline control (BA = 13 ± 3 vs. 26 ± 5%, and BA-Br = 28 ± 8 vs. 44 ± 9%) and in the hemolysate-injected ZL rats compared with their saline control (BA = 24 ± 4 vs. 32 ± 4%; but not BA-Br = 39 ± 6 vs. 59 ± 9%). No significant difference in sodium nitroprusside reactivity was observed. Western blot analysis of the BA showed a lower baseline level of neuronal nitric oxide synthase expression and an enhanced cyclooxygenase-2 level in the hemolysate-injected ZO animals. In summary, cerebrovascular reactivity to both endothelium-dependent and -independent stimuli is severely compromised by SAH in IR animals.

Insulin resistance (IR), a normally “silent” and undetected predecessor of type II diabetes, is a major risk factor for the development of cerebral vascular disease and neurological pathologies such as strokes. There is an increased prevalence of both ischemic and hemorrhagic strokes in IR individuals (4, 14, 45, 56), and stroke patients with type 2 diabetes experience a slower recovery of neurological function and a higher mortality (2, 3, 23, 55). The major underlying basis for augmented neurological damage could be the IR-related dysfunction of the cerebral vasculature. The leptin receptor-deficient Zucker obese (ZO) rat is a commonly used animal model to study IR. The 11–13 wk-old prediabetic ZO rat is characterized by obesity, normal blood pressure, and elevated plasma insulin and lipid levels, without an increase of glucose level. Our laboratory made the original observations that dilator responses of cerebral arteries to physiologically relevant factors are reduced in ZO animals (10, 16, 17, 19). For example, the dilator responses of cerebral arteries mediated by endogenous nitric oxide (NO) production and activation of vascular smooth muscle K+ channels are impaired in ZO rats (10, 17). Moreover, this animal strain appears to be more susceptible to an ischemic brain insult than the Zucker lean (ZL) counterpart (41).

Subarachnoid hemorrhage (SAH) is responsible for 5–10% of all strokes and results in a high rate (50–70%) of mortality (46). The most critical complication of SAH is cerebral vasospasm associated with impaired dilator mechanisms in cerebral arteries. Blood injection into the cisterna magna is a widely used experimental method (47) to investigate the vascular complications of SAH. For several days after the injection of blood, endothelium-derived NO-mediated dilation, the function of several types of K+ channels, and the activity of soluble guanylate cyclase (sGC) dilation have been found to be impaired in the basilar artery (47). However, the relaxation to the ATP-sensitive K+ (KATP) channel opener aprakalim and cromakalim have been shown to be selectively augmented (48, 49, 58).

Several clinical studies focused on the relationship of type 2 diabetes and hyperglycemia to SAH outcome (6, 15, 22); however, to our current knowledge, no studies explored the outcome of SAH in patients with IR or metabolic syndrome compared with patients with no metabolic disease. While the effects of IR or SAH on cerebral arteries have been examined individually and the mechanisms of vascular dysfunction show similarities, we are unaware of studies examining alterations of cerebrovascular responses following intracisternal blood injection in IR rats. Based on the previously introduced studies, it is very probable that the combination of IR and SAH eliminates endothelium-related dilation, whereas it is hard to predict their counteracting effects on KATP channel-mediated relaxation. Therefore, we hypothesized that even a mild SAH creates vasospasm in IR rats and that the dilator responses of the basilar artery and one of its side branches in response to applications of acetylcholine (endothelium-derived NO-mediated dilator), cromakalim (opener of the KATP channel on the vascular smooth muscle), and sodium nitroprusside (direct sGC activator) 24 h after intracisternal injection of heterologous hemolyzed blood in ZO is more severely compromised

Address for reprint requests and other correspondence: D. W. Busija, Dept. of Pharmacology, Tulane Univ., 1430 Tulane Ave., SL 83, New Orleans, LA, 70112-2632 (e-mail: dbusija@tulane.edu).
compared with ZL rats. Furthermore, we quantified the changes of endothelial and neuronal NO synthase (eNOS and nNOS, respectively) levels and cyclooxygenase-2 (COX-2) enzyme expression in the major cerebral arteries, because the change of these enzyme levels may indicate the basis of damaged vascular function (35, 44, 50).

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee at Wake Forest University Health Sciences (Winston-Salem, NC). ZO (11–13 wk old; n = 25) and ZL (11–14 wk old; n = 25) rats (Harlan, Indianapolis, IN) were used for the study.

Intracisternal hemolysate injection. One ZO and one ZL animal were used to obtain 6–6 ml donor blood via cardiac puncture after which the animals were euthanized. The samples were anticoagulated were used to obtain 6–6 ml donor blood via cardiac puncture after 80°C. They were later thawed and used for intracisternal hemolysate injection. The remaining spontaneously breathing rats were anesthetized (5% induction; and 1.7–2.5% maintenance) with isoflurane (Florine) mixed with 40% O2–60% N2O, and the rectal temperature was maintained on 37°C. As previously described (24, 31), Western blot analyses for eNOS, nNOS, and COX-2 were performed using appropriate antibodies. The same amount of extracted protein from whole tissue homogenates of cerebral arteries was loaded for SDS-PAGE/immunoblot analysis. Each immunoband intensity was normalized to the corresponding immunoband intensity of β-actin, which was used as an internal control. The following antibodies were used: anti-eNOS (BD Bioscience), anti-nNOS (Sigma-Aldrich), and anti-COX-2 (Cayman) and a secondary anti-mouse antibody (Jackson Immunoresearch).

Data analysis and statistics. Values are means ± SE. Vascular responses were expressed as the maximal diameter change from baseline in percentage during the drug applications. The program SPSS17 was used for all statistical analysis. The diameters of the basilar arteries were compared with two-way ANOVA. Vascular response data were normalized by logarithmic transformation, which was followed by two-way ANOVA. Survival rates were compared with a Mann-Whitney nonparametric U-test, whereas other physiological parameters were compared with one-way ANOVA. For pairwise comparison, least significant difference post hoc test was applied in all cases.

RESULTS

Experimental conditions during surgical interventions. The intracisternal injection of hemolysate caused a significantly lower survival rate in the OB (57 vs. 100%) than in the LB (62 vs. 82%) rats compared with their saline-injected control group (OS and LS, respectively) (P < 0.05) (Table 1). Isoflurane anesthesia induced elevated plasma glucose levels in all the groups because of its described hyperglycemia-generating effect (33) (Table 1). Under pentobarbital sodium anesthesia, OS and OB animals showed significantly higher glucose levels (162 ± 24 and 179 ± 34 mg/dl, respectively) than the LS and LB groups (103 ± 13 and 82 ± 5 mg/dl, respectively) (P < 0.05), although they were still below the clinically defined “hyperglycemic threshold” (<200 mg/dl) (Table 1). All other parameters were within the physiological range (Table 1).

Baseline diameter of the basilar artery 24 h after intracisternal hemolysate injection. Injection of blood products but not saline into the cisterna magna resulted in the observation of slight discoloration around the basilar artery upon the opening of the dura mater but without the presence of obvious blood clots. The baseline diameters of the basilar arteries were determined before the recording of the dilatory responses by two independent observers with identical results. The diameters in the LB (n = 8; 201 ± 12 μm) and OB (n = 8; 206 ± 8 μm) groups were significantly larger (P < 0.05) than that of the LS (n = 9; 179 ± 8 μm) and OS (n = 10; 183 ± 7 μm) groups (Fig. 1). No difference in the baseline diameter of the side branch was found between the groups (LS = 80.43 ± 15.25, LB = 82.66 ± 20.57, OS = 79.87 ± 12.14, and OB = 87.46 ± 16.96 μm; means ± SD) (Fig. 1).

Vascular responses of the basilar artery and side branch. Acetylcholine (10−6 M) produced a 22 ± 4% dilation of the
basilar artery and 37 ± 7% dilation of the side branch in the 
LS group (Fig. 2, A and B). This response was significantly 
less in the basilar artery of the OS group (9 ± 3%) (P < 0.05) and mildly reduced in the side branch (23 ± 5%).

Table 1. Physiological parameters of the experimental groups during intracisternal injection and during vascular reactivity measurements

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Weight at the injection, g</th>
<th>SR, %</th>
<th>CSF Withdrawn, μl</th>
<th>Blood Sugar Before Injection, mg/dl</th>
<th>Blood Sugar Before Vascular Experiment, mg/dl</th>
<th>MAP, mmHg</th>
<th>pH</th>
<th>Pco2, mmHg</th>
<th>P02, mmHg</th>
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<tbody>
<tr>
<td>LS</td>
<td>11</td>
<td>303 ± 22</td>
<td>82</td>
<td>180 ± 12</td>
<td>178 ± 10</td>
<td>103 ± 13</td>
<td>98 ± 5</td>
<td>7.40 ± 0.01</td>
<td>35 ± 0.8</td>
<td>167 ± 3</td>
</tr>
<tr>
<td>LB</td>
<td>13</td>
<td>340 ± 23</td>
<td>62</td>
<td>158 ± 17</td>
<td>206 ± 22</td>
<td>82 ± 5</td>
<td>99 ± 8</td>
<td>7.38 ± 0.01</td>
<td>38 ± 1.2</td>
<td>167 ± 2</td>
</tr>
<tr>
<td>OS</td>
<td>10</td>
<td>442 ± 14*</td>
<td>100</td>
<td>121 ± 11*</td>
<td>170 ± 17</td>
<td>162 ± 24</td>
<td>105 ± 4</td>
<td>7.38 ± 0.01</td>
<td>37 ± 0.9</td>
<td>166 ± 2</td>
</tr>
<tr>
<td>OB</td>
<td>14</td>
<td>420 ± 19*</td>
<td>57*</td>
<td>98 ± 12*</td>
<td>208 ± 19</td>
<td>179 ± 34</td>
<td>101 ± 6</td>
<td>7.37 ± 0.02</td>
<td>38 ± 1.0</td>
<td>164 ± 3</td>
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Parameters of the Superfused aCSF

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<thead>
<tr>
<th></th>
<th>n</th>
<th>pH</th>
<th>Pco2, mmHg</th>
<th>P02, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS</td>
<td>8</td>
<td>7.40 ± 0.02</td>
<td>42 ± 1.1</td>
<td>128 ± 9</td>
</tr>
<tr>
<td>LB</td>
<td>8</td>
<td>7.45 ± 0.01</td>
<td>38 ± 1.3</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>OS</td>
<td>9</td>
<td>7.42 ± 0.01</td>
<td>38 ± 1.0</td>
<td>151 ± 15</td>
</tr>
<tr>
<td>OB</td>
<td>10</td>
<td>7.42 ± 0.01</td>
<td>38 ± 1.4</td>
<td>121 ± 16</td>
</tr>
</tbody>
</table>

Values are means ± SE. LS, Zucker lean (ZL) saline-injected group; LB, ZL blood-injected group; OS, Zucker obese (ZO) saline-injected group; OB, ZO blood-injected group; SR, survival rate; aCSF, artificial cerebrospinal fluid; MAP, mean arterial blood pressure. *P < 0.05, ZO vs. ZL.
group both in the basilar artery (7 ± 2%) and in the side branch (11 ± 3%) \( (P < 0.05). \) The impaired dilatation of the OB group was more pronounced in the basilar artery (4 ± 2%) but was also significant in the side branch (12 ± 3%) \( (P < 0.05). \)

Cromakalim at \( 10^{-7} \text{ M} \) and \( 10^{-6} \text{ M} \) dilated both the basilar artery (Fig. 3, A–C) and the side branch (Fig. 3, B–D) of the LS group in a dose-dependent fashion. The vascular responses to the lower dose \( (10^{-7} \text{ M}) \) of cromakalim were significantly reduced in the basilar artery of the ZO groups (OS and OB) compared with the counterpart ZL groups (LS and LB) \( (P < 0.05) \), whereas the hemolysate injection did not change the basilar artery dilation in either the LB \( (11 ± 4\% \) versus the LS \( (10 ± 5\% \) group and in the OB \( (4 ± 2\% \) versus the OS \( (5 ± 1\% \) group (Fig. 3, A and B). A similar but not significant tendency was found for the response of the side branch. The OS \( (9 ± 1\% \) and OB \( (11 ± 4\% \) groups showed less relaxation compared with the LS \( (16 ± 2\% \) and LB \( (18 ± 6\% \) groups, whereas no change was found in the side branch reactivity after hemolysate injection to low-dose cromakalim. In contrast, the basilar artery response to the higher dose \( (10^{-6} \text{ M}) \) of cromakalim was significantly blunted in the OB \( (13 ± 3\% \) compared with the OS \( (26 ± 5\% \) group \( (P < 0.05) \), whereas the dilatation in the LB group \( (24 ± 4\% \) was not considerably less than in the LS group \( (32 ± 4\% \). The side branch also showed a significantly reduced relaxation in the obese groups (OS \( = 44 ± 9\) and OB \( = 28 ± 8\% \) versus the lean groups (LS = 59 ± 8, and LB = 39 ± 6\% \) \( (P < 0.05) \), whereas the response of the side branches remained unaltered by the hemolysate injection.

While there was a tendency for a reduced vascular responsiveness to sodium nitroprusside in the OS and OB groups compared with the LS and LB groups, respectively, and the power of the statistical analysis was low (0.055–0.341), the difference was not significant (Fig. 4).

**Protein expression of the cerebral vessels after intracisternal hemolysate injection.** There were no differences in eNOS levels among the four groups (Fig. 5A). The eNOS enzyme levels in the LS and OS animals were the same (100 ± 12 and 111 ± 12\%, respectively), and the hemolysate injection did not change the eNOS expression \( (109 ± 17 \text{ for LB and } 122 ± 9\% \text{ for OB group}) \). However, nNOS expression was significantly reduced in the OS \( (n = 3; 63 ± 6\% \) compared with the LS \( (n = 3; 100 ± 9\% \) groups \( (P < 0.01) \) Intracisternal hemolysate injection had no effect on the vascular nNOS levels in the LB \( (n = 4; 104 ± 11\% \) and the OB \( (n = 4; 66 ± 10\% \) groups (Fig. 5B).

The COX-2 expression in the cerebral vessels of LS \( (100 ± 14\%; \ n = 4 \) and OS \( (96 ± 16\%; \ n = 4 \) rats was similar (Fig. 5C). The LB showed only a modest increase in vascular COX-2 expression \( (136 ± 17\%; \ n = 4 \) whereas the OB rats presented a significant twofold elevation in the protein level \( (n = 5; 205 ± 35\% \) \( (P < 0.05) \).

![Fig. 3. Dose-dependent responses of the BA (A–C) and the side branch (B–D) of ZO and ZL rats to \( 10^{-7} \text{ M} \) (A and B) and \( 10^{-6} \text{ M} \) (C and D) cromakalim 24 h after intracisternal hemolysate or saline injection. Data are expressed as percent changes from baseline diameter (means ± SE). LS \( = 8 \), LB \( = 8 \), OS \( = 10 \), and OB \( = 7 \) groups are shown. \# \( P < 0.05 \text{, blood vs. saline; } \# P < 0.05 \text{ obese vs. lean.} \)](http://ajpheart.physiology.org/)

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The major finding of the study is that the adverse effects of SAH on cerebral vascular dilator responses are exacerbated in insulin-resistant animals. Thus both endothelial-dependent and independent responses are more impaired following a single injection of hemolyzed blood into the cisterna magna in ZO compared with ZL rats. While the mechanisms involved are not entirely clear, we suggest that an increase in COX-2 levels is a potential contributor for impaired cerebral vascular responsiveness. Nonetheless, the translational implication is that insulin-resistant individuals are at risk for exaggerated negative effects of perivascular blood around cerebral resistance vessels, and this factor should be taken into consideration during treatment of people suffering from SAH. The single injection of 300 μl heterologous hemolysate 1 day before examination did not create vasospasm in the basilar artery of ZO and ZL rats but rather slightly increased the baseline diameter. We have observed similar results in a previous study in piglets, where a single injection of blood products around the cortical arteries did not induce vasospasm but inhibited dilator responses to arterial hypercapnia and hypotension but not to isoproterenol.

Delayed vasoconstriction is a widely known and severe complication of SAH (32, 34, 46, 47, 54, 57) in people, but its presence in experimental animals is dependent on technical issues such as timing and number of blood injections. For example, a double injection model using fresh, nonhemolyzed autologous blood is normally required to induce basilar artery vasospasm in rats and dogs (25–28, 39). However, vasospasm takes several days to develop, and it is possible, but unexplored, that cerebral vascular responses in people are impaired even during the prespasm period. Furthermore, the reduced baseline diameter of the vasospastic arteries as well as the underlying pathology in SAH would be expected to exacerbate the derangement of the vascular effects of the basilar to dilator agents.

Acetylcholine elicited smaller responses in the OS than in the LS rats, but the responses to the NO donor sodium nitroprusside were not statistically different among the four groups. Although no changes were seen in eNOS abundance and the nNOS abundance was lower, the reduced vasodilation, dependent on endogenous NO, is likely due to reduced NO synthase activity or low NO bioavailability via the well-known NO scavenging action of oxygen free radicals (19, 21). Impaired function and expression of acetylcholine receptors on vascular endothelial cells or the altered reactivity of smooth muscle cells to the released NO may be responsible for the difference of acetylcholine-mediated dilation in the ZO versus the ZL rats in SAH, but further systematic investigations are needed to clarify the molecular counteraction of IR and SAH. However, this is the first study to show that the reduced dilator response of the cerebral arteries of ZO rats to acetylcholine is correlated with a lower expression of nNOS. Our results are supported by Cellek et al. (12) who have previously shown that nNOS-expressing perivascular nerves around the basilar artery are progressively degenerated in streptozotocin-induced diabetic rats. While nNOS is normally localized to perivascular nerves associated with cerebral arteries and has several well-defined functions, nNOS has been shown to compensate for reduced NO and thereby restore normal NO-dependent dilation in eNOS knockout mice (36, 37). In addition, some investigators have presented evidence that the specific inhibitor of nNOS, 7-nitro indazole, reduces the dilation of the basilar artery to acetylcholine (7). Nonetheless, our results indicate that endothelium-linked dilator responses are dramatically reduced by a single exposure of 300 μl perivascular hemolysate and that this impaired dilation is greater in ZO than in ZL rats. The sodium nitroprusside response was not significantly different in the ZO compared with the ZL rats and was not affected by a single hemolysate injection. This finding suggests that dilation to NO donors is not severely compromised by SAH in IR. Based on previous observations from our laboratory, it is known that the response to sodium nitroprusside remains intact in IR rats (19, 20). A previous study on isolated canine basilar arteries after double injection of blood into the cisterna magna showed that besides reduced sGC expression and lower cyclic guanosine monophosphate production, the response to sodium nitroprusside is preserved via Ca2+-activated K+ channels (40). Despite this, we found that the function of these channels is impaired in IR rats (16, 18). It is important to note, however, that because of the low statistical power of our sodium nitroprusside responses, it is possible that using a different dose of sodium nitroprusside, applying repeated rather than only one hemolysate injection or making measurements at a later time period,
might show a significant reduction in sodium nitroprusside response in the ZO rats after SAH.

The \( K_{\text{ATP}} \) channel-activated dilation with cromakalim was not preserved or enhanced after SAH, as previously described (48, 49) in normal rats, but was rather reduced in both ZO and ZL rats. The decrease in vascular reactivity was significantly greater in the ZO than in the ZL rats. Similar results were obtained from the measurement of side branch reactivity, but the extent of vascular dysfunction was more moderate. These findings suggest that the impairment of vascular responses to \( K_{\text{ATP}} \) channel activators by the hemolysate injection is more severe in major cerebral vessels than in smaller cerebral arteries. Whereas most \( K^+ \) channel-mediated responses are impaired after SAH except for the \( K_{\text{ATP}} \) channels, which are selectively enhanced, the pharmacological activation of the \( K_{\text{ATP}} \) channel with either endogenous substances or synthetic analogs is a favorable approach to treat cerebral vasospasm (1, 30, 39, 51, 52, 58). Although we did not directly test \( K_{\text{ATP}} \) channel function on vasospastic vessels, these therapies might be of lower efficacy in the presence of IR based on our data. The mechanism of \( K_{\text{ATP}} \) channel dysfunction in IR is most likely related to the effect of enhanced reactive oxygen species production (9, 18, 19).

No difference was observed between the baseline COX-2 expression of LS and OS, whereas COX-2 expression increased dramatically in the OB but not in the LB group. The elevation of COX-2 in the cerebral arteries after SAH has been previously described (42, 43, 53). COX-2 produces a variety of vasoactive prostanooids as well as superoxide anion that may have affected the baseline diameter as well as the responsiveness of the basilar artery to both acetylcholine and cromakalim. It appears that a higher expression of COX-2 could be a prominent source of oxygen radicals in the basilar artery during a more moderate exposure to perivascular hemolysate. Further experiments are needed to determine whether the elevated level of COX-2 is correlated with enhanced enzyme function or the higher expression is a compensation for the lower availability of substrates (arachidonic acid and \( \text{O}_2 \)). We have previously shown in newborn pigs that superoxide anion generation related to COX-2 activation is a remarkable factor in producing reduced dilator responses in the cerebral circulation (5). In conclusion, COX-2 may represent a pivotal factor in exaggerating the cerebrovascular dysfunction of the basilar artery and the side branch to SAH.

**Perspective.** Vasospasm represents a well-known, dangerous situation for patients following SAH, but the consequences of perivascular blood before the development of vasospasm are not fully known. Our findings suggest that in this “quiet period” in which blood products are in contact with the exterior of cerebral arteries, before the appearance of vasospasm, the cerebral arteries are already exhibiting reduced responsiveness to both endothelium-dependent and -independent dilator stimuli. Thus the mere presence of perivascular blood products in the absence of confounding variables such as increased intracranial pressure has the potential to impair neurological function by causing an uncoupling between metabolic demand and
cerebral hemodynamics. Furthermore, existing metabolic diseases such as IR are able to exaggerate cerebral vascular dysfunction in SAH, likely because of the mechanisms involving enhanced vascular expression of COX-2. Finally, future clinical studies should pay attention to the presence of IR and metabolic syndrome in the outcome and complications of SAH in patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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