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

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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 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 aprikalim 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.
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 with heparin (1:10). To hemolyze and store the samples, aliquots were put on −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 monitored and maintained on 37°C. The heads were fixed in a stereotaxic frame tilted 30° forward to aid in exposing the cisterna magna. Lubricating ointment gel was applied to the eyes to protect them from drying out, and ears were smeared with lidocaine gel to reduce potential discomfort. The scruff was shaved and disinfected, and after a midline incision, the medial neck muscles were separated and retracted to expose the atlanto-occipital membrane. The tail was clipped to obtain blood, and blood glucose level was analyzed immediately by a handheld blood glucose meter kit (Mckesson True Track). Cerebrospinal fluid (80–200 µl) was then carefully withdrawn from the cisterna magna 2 mm below the occipital bone with a Hamilton syringe. A 30-gauge needle connected to a catheter was introduced 2 mm deep into the same hole, and 300 µl heterologous hemolyzed blood was injected into the cisterna magna with an infusion pump for 30 min (10 µl/min).

Subsequently, the rats were kept in the same position for an additional 30 min to facilitate the settling of blood products in the basal cisterns, and the catheter was removed and the muscle and skin were sutured. To prevent volume depletion, the rats received 2 ml/kg saline subcutaneously and returned to their cages for 24 h with free access to food and water. The following experimental groups were created: ZL rats with intracisternal saline (LS) or blood (LB) injection and ZO rats with saline (OS) or blood (OB) injection.

Vascular reactivity measurement. Twenty-four hours after the injection, the animals were reanesthetized with pentobarbital sodium (90–100 mg/kg ip) and mechanically ventilated via tracheotomy with O2-enriched room air. The end-tidal partial pressure of CO2 was continuously recorded (Microcapnograph CI240, Columbia Instruments) and was kept between 38–40 mmHg. The body temperature was monitored with a rectal probe and maintained around 37°C with a heating blanket. A catheter was placed into the femoral artery to monitor arterial blood pressure and to obtain arterial blood samples. The femoral vein was also cannulated to supplement anesthetics (20 mg·kg−1·h−1) with an infusion pump. The rats were arranged in a supine position and the heads secured into place with a stereotaxic apparatus. After carefully separating and retracting the neck muscles in the midline, we performed a ventral craniotomy 2–3 mm in diameter with a drill over the base of the skull. Inflow and outflow ports were placed above the window to allow the superfusion of artificial cerebrospinal fluid at a rate of 3 ml/min. The membranes were then removed from over the basilar artery with fine pincers and a bent 30-gauge needle. The basilar artery and a side branch were visualized by a surgical microscope equipped with a charge-coupled device camera connected to a computer, and the diameters were analyzed using the Scion Image Software (Scion, Frederick, MD, USA). The dilator responses of the basilar artery and the side branch to acetylcholine (10−6 M), cromakalim (10−7 M, 10−6 M), and sodium-nitroprusside (10−7 M) were recorded at a sampling rate of 1 image/2 s. Acetylcholine was purchased from Sigma (St. Louis, MO), and cromakalim was bought from Tocris (Ellisisville, MO). At the end of the experiment, the animals were euthanized with an overdose of anesthesia, and the main cerebral arteries (basilar artery and 2 anterior, 2 media, and 2 posterior cerebral arteries) were cleaned, removed, and stored at −20°C in an extraction buffer for Western blot analysis.

Western blot analysis. 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 homogonates 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 matter 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% 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%).

Hemolysate injection diminished the response of the LB artery and 37% 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%).

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.

### 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>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>Parameters of the Superfused aCSF</th>
<th>Blood Gases Before Vascular Experiment</th>
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<td>pH</td>
<td>PO2, mmHg</td>
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<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>
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<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>
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<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>
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<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>
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Parameters of the Superfused aCSF:

- pH
- PO2, mmHg
- PO2, mmHg

Blood Gases Before Vascular Experiment:

- pH
- PO2, mmHg
- PO2, mmHg

*P < 0.05, blood vs. saline.

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Fig. 1. The internal diameter of the basilar artery (BA) 24 h after intracisternal hemolysate injection in Zucker lean (ZL) and Zucker obese (ZO) rats. LS, ZL saline-injected group (n = 8), LB, ZL blood-injected group (n = 9), OS, ZO saline-injected group (n = 8), and OB, ZO blood-injected group (n = 10) are shown. *P < 0.05, blood vs. saline.

Fig. 2. Acetylcholine (10^-6M) response of the BA (A) and its side branch (B) 24 h after intracisternal hemolysate or saline injection in ZO and ZL rats. Data are expressed as percent change from baseline diameter (means ± SE). LS (n = 8), LB (n = 9), OS (n = 9), and OB (n = 8) groups are shown. *P < 0.05, blood vs. saline; #P < 0.05, obese vs. lean.
group both in the basilar artery (7 ± 2%) and in the side branch (11 ± 3%) \( (P < 0.05) \). The impaired dilation 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 \( (OS \text{ and OB}) \) \( (P < 0.05) \) compared with the counterpart ZL groups \( (LS \text{ and LB}) \), whereas the hemolysate injection did not change the basilar artery dilation in either the LB \( (11 \pm 4\%) \) versus the LS \( (10 \pm 5\%) \) group and in the OB \( (4 \pm 2\%) \) versus the OS \( (5 \pm 1\%) \) group \( (P < 0.05) \). A similar but not significant tendency was found for the response of the side branch. The OS \( (9 \pm 2\%) \) and OB \( (11 \pm 4\%) \) groups showed less relaxation compared with the LS \( (16 \pm 2\%) \) and LB \( (18 \pm 6\%) \) groups, whereas no change was found in the side branch reactivity after hemolysate injection to low-dose cromakalim. Intracisternal hemolysate injection had no effect on the vascular nNOS levels in the LB \( (n = 4; 104 \pm 11\%) \) and the OB \( (n = 4; 66 \pm 10\%) \) groups (Fig. 5B).

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

**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 \( (n = 8) \), LB \( (n = 8) \), OS \( (n = 10) \), and OB \( (n = 7) \) groups are shown. \*\( P < 0.05 \), blood vs. saline; \#\( P < 0.05 \) obese vs. lean.
The major finding of the study is that the adverse effects of SAH on cerebral 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 associated with cerebral arteries and has several well-defined 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 in 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 neurons 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 effects on cerebral vascular tone (11, 13, 29, 38), 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 dilatation 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 (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, applied 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 prostanoids 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 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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