Temporal profile and significance of metabolic failure and trophic changes in the canine cerebral arteries during chronic vasospasm after subarachnoid hemorrhage

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To investigate the pathogenetic significance of metabolic failure observed in spastic cerebral arteries after subarachnoid hemorrhage (SAH), the temporal profile of alterations in the arterial content of high-energy phosphates was studied. A canine model of double hemorrhage was used. Constriction of the basilar artery was measured angiographically on Days 3, 5, 7, and 14 after SAH in separate groups of animals. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), guanosine triphosphate (GTP), guanosine diphosphate, creatine phosphate (CrP), and creatinque (Cr) levels in the arteries were assayed using high-performance liquid chromatography. A time-dependent development of angiographic spasm was confirmed. A mild vasospasm was seen in the group studied 3 days after SAH, progressed in the Day 5 group, remained comparably severe in the Day 7 group, and resolved partially in the Day 14 group. The content of high-energy phosphates (ATP, GTP, and CrP) declined rapidly over the course of the study, and a significant reduction in ATP, GTP, and CrP was observed in the Day 3 group. Levels of ATP and CrP decreased further in the Day 5 and 7 groups. The decrement in GTP was completed in the early phase; a significant reduction took place in the Day 3 group, with no progression thereafter and no recovery though Day 14. Total adenylate (ATP + ADP + AMP) and total creatine (Cr + CrP) content diminished markedly over the course of the study. These results indicate that metabolic failure and trophic disturbance in the cerebral artery occurs with a rapid onset following SAH and progresses in close association with the development of vasospasm, suggesting a significant causal relationship with the pathogenesis.

KEY WORDS  * adenosine triphosphate  * guanosine triphosphate  * metabolism  * creatine phosphate  * cerebral vasospasm  * subarachnoid hemorrhage  * dog

Despite numerous efforts to elucidate the pathogenetic mechanism of chronic cerebral vasospasm after subarachnoid hemorrhage (SAH), the true nature of pathological arterial constriction has yet to be clarified. Various aspects of phenomena associated with the prolonged constriction, such as changes in pharmacological responsiveness, alterations in mechanical properties of the arterial wall, and immunological reactions, have been studied. Recently, an observation was made that high-energy phosphate content of the wall of the canine basilar artery is markedly diminished during chronic vasospasm. The finding indicates that the arterial wall is in a state of metabolic failure and signifies a possibility that cellular homeostasis of smooth muscle is deranged during the pathological prolonged contraction. The present study was designed to assess the significance of metabolic alteration of the vasculature in the pathogenesis of cerebral arterial spasm. The temporal profile of the metabolic derangement/trophic alterations in spastic cerebral arteries following SAH was studied, and the course was analyzed in correlation with the development of angiographic vasospasm.

Materials and Methods

Animal Model and Groups

The canine model of "double" SAH was used in the present study. Twenty mongrel dogs of either sex, weighing 8 to 13 kg each, were randomly allocated to five groups of four animals each: a control group and groups studied on Day 3, Day 5, Day 7, or Day 14 after SAH. The mean body weight of the animals was comparable between each group. The care of the animals and procedures in the study complied with the "Principles of Laboratory Animal Care" and the "Guide for the Care and the Use of Laboratory Animals" published by the National Institutes of Health.

With the dogs under general anesthesia (pentobarbital, 15 mg/kg, and thiopental, 15 to 25 mg/kg) and mechanical ventilation, vertebral angiography was performed to measure the size of the basilar artery. Arterial blood gas levels were monitored to safeguard against
TABLE 1
Content of total protein, total adenylate, and total creatine in each animal group

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Protein Mean (µg/segment)</th>
<th>No. of Segments</th>
<th>Total Adenylate Mean (nmoles/segment)</th>
<th>No. of Segments</th>
<th>Total Creatine Mean (nmoles/segment)</th>
<th>No. of Segments</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>87 ± 5</td>
<td>16</td>
<td>3.9 ± 0.2</td>
<td>16</td>
<td>15.4 ± 0.7</td>
<td>16</td>
</tr>
<tr>
<td>Day 3</td>
<td>70 ± 5</td>
<td>16</td>
<td>2.3 ± 0.2†</td>
<td>16</td>
<td>6.5 ± 0.5†</td>
<td>8</td>
</tr>
<tr>
<td>Day 5</td>
<td>74 ± 4</td>
<td>16</td>
<td>1.9 ± 0.1†</td>
<td>16</td>
<td>6.6 ± 0.5†</td>
<td>16</td>
</tr>
<tr>
<td>Day 7</td>
<td>83 ± 5</td>
<td>16</td>
<td>1.9 ± 0.1†</td>
<td>16</td>
<td>5.6 ± 0.3†</td>
<td>12</td>
</tr>
<tr>
<td>Day 14</td>
<td>67 ± 3</td>
<td>16</td>
<td>1.4 ± 0.1†</td>
<td>16</td>
<td>4.3 ± 0.4†</td>
<td>16</td>
</tr>
</tbody>
</table>

* Means are expressed ± standard error of the mean. Total adenylate = adenosine triphosphate + adenosine diphosphate + adenosine monophosphate. Total creatine = creatine + creatine phosphate.
† Significant difference compared with the control group (p < 0.05).

Angiographic vasospasm after two injections of autologous venous blood (7 to 10 ml each) into the cisterna magna on Days 0 and 2 after subarachnoid hemorrhage in dogs. The control group underwent angiography on Days 0 and 7 but no cisternal injection. The degree of vasospasm is expressed as the ratio of the cross-sectional area to that on the baseline angiogram. A vertical bar indicates the standard error of the mean for four segments from each of four animals in each group (16 segments). S = significant difference between the two compared sets of data (p < 0.05, analysis of variance); NS = no statistically significant difference.

Angiographic Analysis
For quantification of the degree of vasospasm, the angiograms were magnified and the images were recorded and digitized using a video transfer system coupled with a personal computer and an image analyzer. The angiographic image of a basilar artery was divided into four segments, and the mean cross-sectional area was calculated for correlation with the content of high-energy phosphates in each segment. The degree of narrowing was calculated as the ratio of the cross-sectional area to that on the baseline angiogram.

Determination of High-Energy Phosphate Content
Levels of adenosine phosphates (adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP)), guanosine phosphates (guanosine triphosphate (GTP) and guanosine diphosphate (GDP)), and creatine (Cr) and creatine phosphate (CrP) contents in the basilar artery were measured using an identical method to that described in a previous study. Following sacrifice of the animal, the brain and the cervical cord were promptly dissected, stored at 0°C in a modified Krebs-Ringer solution containing no glucose (control solution; composition (mM): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, and bubbled with 95% N2 and 5% CO2. Subarachnoid clot on the adventitial surface of the artery and in the lumen was removed meticulously under microscope. The basilar arteries were divided into four segments and trimmed to 4 mm in length. The dissection and preparation were performed in the control solution cooled to 0°C. The arterial segments were rapidly frozen in liquid nitrogen for 60 seconds and moved into ice-cold perchloric acid (0.42 M, 500 µl).

Homogenization was performed using a glass homogenizer* in an ice-cold water bath. Protein content was precipitated with the alkali KOH (1 M, 150 to 180 µl) and, after adjusting pH to between 7 and 8 with 1 M HCl, the samples were centrifuged at 2000 G for 4 minutes at 0°C. Twenty microliters of the supernatant was injected into the high-performance liquid chromatography system. Loss of high-energy phosphates during sample preparation was demonstrated to be negligible in a previous study. The high-performance liquid chromatography system consisted of a solvent delivery system, a multiphotodiode-array detector, and

* Homogenizer manufactured by Corning Glass Works, Corning, New York.

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FIG. 1. Graph demonstrating temporal profile of angiographic vasospasm after two injections of autologous venous blood (7 to 10 ml each) into the cisterna magna on Days 0 and 2 after subarachnoid hemorrhage in dogs. The control group underwent angiography on Days 0 and 7 but no cisternal injection. The degree of vasospasm is expressed as the ratio of the cross-sectional area to that on the baseline angiogram. A vertical bar indicates the standard error of the mean for four segments from each of four animals in each group (16 segments). S = significant difference between the two compared sets of data (p < 0.05, analysis of variance); NS = no statistically significant difference.

fluctuation in the size of the cerebral artery due to variations in pCO2. Following angiography, the animals in the SAH groups underwent a percutaneous injection of autologous venous blood (7 to 10 ml) into the cisterna magna on Day 0 and again on Day 2. Technical details of the procedure have been described in previous reports. The animals in the Day 3 group underwent repeat angiography on Day 3 and were then sacrificed with an intravenous dose of sodium pentobarbital (30 mg/kg). Similarly, the dogs in the Day 5, 7, and 14 groups underwent repeat angiography and were sacrificed on Day 5, 7, or 14, respectively. The control group underwent angiography but no injection into the cisternal magna, and angiography was repeated on Day 7.

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Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Segments</th>
<th>ATP:ADP</th>
<th>GTP:GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>16</td>
<td>1.78 ± 0.07</td>
<td>3.07 ± 0.26</td>
</tr>
<tr>
<td>Day 3</td>
<td>16</td>
<td>1.53 ± 0.06</td>
<td>2.31 ± 0.22</td>
</tr>
<tr>
<td>Day 5</td>
<td>16</td>
<td>1.04 ± 0.081</td>
<td>2.01 ± 0.22†</td>
</tr>
<tr>
<td>Day 7</td>
<td>16</td>
<td>1.09 ± 0.06†</td>
<td>2.26 ± 0.13</td>
</tr>
<tr>
<td>Day 14</td>
<td>16</td>
<td>0.91 ± 0.07†</td>
<td>2.34 ± 0.24</td>
</tr>
</tbody>
</table>

* Data are expressed ± standard error of the mean. ATP = adenosine triphosphate, ADP = adenosine diphosphate, GTP = guanosine triphosphate, GDP = guanosine diphosphate.
† Significant difference compared with the control group (p < 0.05).

an ultraviolet absorbance detector, a plotter, and a 15-cm reversed-phase column with C-18 coating (TSK gel octadecyl silane 80 trimethylsilane, 15 cm × 4.6 mm, particle size 5 μ) with a 3-cm guard column.§

The mobile phase for measurement of purine nucleotide was a solution containing 215 mM KH$_2$PO$_4$, 3.45 mM tetrabutylammonium hydrogen sulfate (TBAHS), and 3.5% acetonitrile,† that for measurement of Cr and CrP consisted of 14.7 mM KH$_2$PO$_4$ and 2.36 mM TBAHS.‡ The solutions were prepared and degassed before each assay, and the flow rate was maintained at 1.2 ml/min. For identification of peaks in the samples, a spectrophotometric detector was set to scan from 190 to 350 nm, and the absorbance spectrum was compared to the standards. To confirm the identification of the retention time, a known amount of the standards was added to the samples and augmentation of a corresponding peak was proved. After identification, the wavelength was set at 260 nm for detecting adenosine and guanosine phosphates and at 200 nm for detecting Cr and CrP.

Measurement of Protein Content

The precipitated protein content of each arterial sample was resuspended, solubilized with 1% sodium dodecyl sulfate,† and thoroughly homogenized. Total protein content of the solution was assayed via spectrophotometry using a bicinocochinic acid protein assay reagent kit.§

Data Analysis

The data are expressed as means ± standard error of the means; the individual data for purine nucleotides and CrP are expressed as nmole/mg of protein. Total adenylate (ATP + ADP + AMP), total Cr (Cr + CrP), and ratios of ATP:ADP and of GTP:GDP were calculated for each sample. For statistical comparison, one-way analysis of variance was used first. If a significant difference was detected between the groups (p < 0.05), individual sets of data were further compared with critical values of modified t-test statistics obtained using the Bonferroni method. In all cases, p values less than 0.05 were considered to be statistically significant. For evaluation of the correlation between angiographic narrowing and phosphate contents in each segment, linear regression analysis was used. Nonparametric analysis of the correlation between the mean values of these two parameters in each group was performed using Spearman’s rank test.

Results

angiographic Vasospasm

Narrowing of the basilar artery was induced reliably in the SAH groups. A moderate reduction in the cross section of the basilar artery was observed in the group studied 3 days after SAH as compared to the control group (Fig. 1). In the Day 5 group, arterial spasm was significantly more intense than in the Day 3 group. Between Days 5 and 7, no progression in the degree of spasm was observed, indicating that the course had reached its plateau. Partial but significant resolution of the narrowing was observed in the Day 14 group (Fig. 1).

Total Protein Content

In the groups studied on Day 3, 5, 7, and 14 after SAH, the mean total protein content of the 4-mm basilar artery segment was slightly decreased from the value in the control group (Table 1). However, no significant differences were detected when comparisons were made between any two groups.

High-Energy Phosphate Content

A significant decrease in the high-energy phosphate levels was observed in the groups undergoing SAH. The ATP content (Fig. 2 left) decreased significantly in the Day 3 group as compared to the control group, and the value was further decreased in the Day 5 group. No difference in the ATP content was seen between the Day 5 and Day 7 groups, and the value did not recover in the Day 14 group when compared to the Day 5 or Day 7 group. A decrease in the content of GTP took place in the early phase of the course (Fig. 2 center); a marked reduction in the value was seen in the Day 3 group and the values in the Day 5, 7, and 14 groups did not differ significantly from those of the Day 3 group. The CrP content (Fig. 2 right) was significantly reduced in the Day 3 group and decreased further over the course, but no significant change was detected among the Day 3, 5, and 7 groups. The CrP content was virtually unchanged in the Day 14 group in comparison to the Day 7 group.

The ATP:ADP ratio (Table 2) was smaller in the group studied 5 days after SAH than in the control group. No difference was seen between the Day 5 and Day 7 groups, and the ratio did not recover in the Day 14 group. A reduction in the GTP:GDP ratio was found in the SAH groups, and a significant difference was found in the value for the Day 5 group compared with that in the control group.

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subarachnoid hemorrhage. The phosphate content is normalized to the total amount of protein in each sample tissue (nmoles/mg protein). A vertical bar indicates the standard error of the mean for four samples from each of four animals in each group (16 samples). S = significant difference between the two compared sets of data (p < 0.05, analysis of variance); NS = no statistically significant difference. **Left:** Content of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). **Center:** Content of guanosine triphosphate (GTP) and guanosine diphosphate (GDP). **Right:** Content of creatine phosphate (CrP).

**Fig. 2.** Graphs showing the course of alteration in the content of high-energy phosphates in the canine basilar artery after subarachnoid hemorrhage. The phosphate content is normalized to the total amount of protein in each sample tissue (nmoles/mg protein). A vertical bar indicates the standard error of the mean for four samples from each of four animals in each group (16 samples). S = significant difference between the two compared sets of data (p < 0.05, analysis of variance); NS = no statistically significant difference. **Left:** Content of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). **Center:** Content of guanosine triphosphate (GTP) and guanosine diphosphate (GDP). **Right:** Content of creatine phosphate (CrP).

**Fig. 3.** Graph showing the relationship between angiographic vasospasm and the content of adenosine triphosphate (ATP) after subarachnoid hemorrhage (SAH) in dogs. The ratio of the cross-sectional area of the basilar artery on the angiograms obtained on Day 0 (abscissa) and on the day of sacrifice and the ATP content normalized to the total protein content (nmoles/mg protein, ordinate) are plotted for each segment (four segments from each animal). Crosses indicate the mean ± standard error of the mean of the two parameters in each group. A significant rank correlation was detected in the mean values in the control group and groups studied on Days 3, 5, and 7 after SAH (p < 0.05, Spearman's rank test). A positive linear correlation was observed for the individual samples in the first four groups (solid line, slope = 17.7, r² = 0.49, p < 0.001). The ATP level progressively decreased through Day 14, despite a partial resolution of the angiographic vasospasm. A negative linear correlation (dotted line, slope = -6.7, r² = 0.14, p < 0.05) was detected between the two parameters for the samples in the Day 7 and 14 groups.

**Total Adenylate and Total Creatine Content**

The total adenylate content (AMP + ADP + ATP) and total Cr value (CrP + Cr) were decreased in the SAH groups (Table 1). A significant reduction in the total adenylate content was found in the group studied 3 days after SAH, and the content decreased further in the Day 5 group. The values were comparable between the Day 5 and Day 7 groups. The content was lower in Day 14 group as compared to the Day 7 group. The total Cr content decreased markedly in the Day 3 group, and it remained comparable in the Day 5 and 7 groups. No recovery of total Cr was seen in the Day 14 group.

**Correlation Between Angiographic Spasm and High-Energy Phosphate Levels**

The ATP content decreased in parallel with the progression of arterial narrowing in the control group and in the groups studied 3, 5, and 7 days after SAH (Fig. 3). A significant rank correlation between the mean values of the two parameters was detected in the four groups (p < 0.05, Spearman's rank test). A positive linear correlation was detected between the individual values of ATP and the degree of angiographic spasm in the arteries of the control group and in the groups studied 3, 5, and 7 days after SAH when combined (p < 0.001, slope = 17.7, Fig. 3). A negative linear correlation was observed between these values in the Day 7 and 14 groups (p < 0.05, slope = -6.65, Fig. 3), where ATP levels progressively decreased despite partial resolution of angiographic vasospasm.

**Discussion**

The course of angiographic vasospasm measured in this study is consistent with that reported from another
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Laboratory using the canine model of double hemorrhage; a mild decrease in the diameter of the basilar artery was observed on Day 3, the narrowing progressed and reached a plateau between Days 5 and Day 7, and a partial resolution was observed on Day 14. A previous study using the identical model demonstrated a significant decrease in the high-energy phosphate content in the spastic basilar artery 1 week after hemorrhage. The present study confirmed that finding and further elucidated the temporal profile of metabolic impairment in association with the angiographic spasm.

Time Course of High-Energy Phosphate Reduction

A progressive decrease in the high-energy phosphate levels was observed concomitant with the development of vasospasm. In the course of reduction, however, there were some differences in behavior among ATP, GTP, and CrP. The ATP content decreased to approximately 70% of the control value in the first 3 days after initial hemorrhage. It decreased further to 40% between Day 5 and Day 7, changes corresponding to the course of angiographic narrowing. There was a significant period of positive interaction between the severity of angiographic spasm and the reduction in ATP content when compared among the control group and the Day 3, 5, and 7 groups.

In comparison to the course of ATP, GTP decreased more rapidly; it was reduced to approximately 60% of the control value by Day 3, and reduction was small thereafter. Guanosine triphosphate is the substrate for enzymatic production of cyclic GMP, a ubiquitous and critical second messenger for smooth-muscle relaxation. Originally, a finding of impaired cyclic production in the spastic artery led us to study changes in GTP and other high-energy phosphates.

Creatine phosphate, the energy reserve compensating for the decrease in ATP and GTP, decreased to 60% of the control value by Day 3 and progressively decreased from Day 5 to Day 7, when the value reached a plateau. Approximately 60% of the total change in the CrP level took place in the first 3 days.

Significance of High-Energy Phosphate Reduction

These findings indicate that a significant reduction of the high-energy phosphates takes place in the early phase after SAH, when constriction of the artery is still developing. The phosphate levels were expressed in values normalized per total protein content. The protein content in each 4-mm segment of the artery did not change significantly over the course of the study. Therefore, the decrease in the levels of high-energy phosphates is not due to changes in the denominator of the normalization process. The progressive decrease in ATP between Day 7 and Day 14 is offset by declines in the protein content, albeit statistically nonsignificant; the reduction in phosphate level would be more remarkable if expressed as an absolute amount in the 4-mm segment. The ATP:ADP and GTP:GDP ratios, markers inherently unaffected by possible changes in the protein volume or composition (muscle vs. non-muscle protein), also decreased over the course of the study. Along with the values of the triphosphates and the triphosphate:diphosphate ratios, the total adenylate content (ATP + ADP + AMP) diminished remarkably. A major change (65%) in the total adenylate content took place by Day 3; the level on Day 3 was 60% of the control and decreased mildly between Day 5 and Day 7. Similarly, the total creatine content (Cr + CrP) decreased to 40% of the control value between Day 0 and Day 3 after SAH; the value continued to decrease subsequently but the reduction over the first 3 days was much larger than the changes thereafter.

These findings indicate that the pathological processes not only reduce the energy-charge state of the arterial wall but also cause a decrease in the total pool of purine nucleotides, signifying a trophic deterioration and affected viability of the smooth-muscle cells. The changes occur in the early phase after SAH followed by the development of vasospasm. The early onset of metabolic impairment together with the significant correlation between the reduction in the phosphates and the degree of vasospasm indicate a close link between the two phenomena and suggest the former as being a contributing cause for the latter.

Metabolic Impairment and Pathogenesis

Several hypothetical mechanisms are conceivable regarding the link between metabolic derangement and constriction of the cerebral artery. The former can lead into dysfunction of any energy-requiring process of cellular homeostasis, including energy-dependent Ca++ extrusion. General failure of metabolism eventually affects membrane integrity, followed by calcium influx and overload. An increase in intracellular Ca++ causes contraction of the contractile protein if the level of the calcium-binding protein calmodulin is maintained. A deficiency of ATP can lead to rigor status in the smooth muscle. It is likely that the observed metabolic impairment affects the smooth-muscle cell population heterogeneously; if some muscle cells are severely affected in a mosaic fashion and the ATP level is close to deficiency status, the smooth-muscle cells can be in rigor. Guanosine triphosphate is the substrate for guanylate cyclase to produce cyclic GMP; recent findings indicate that the second messenger plays a pivotal role in relaxation of vascular smooth muscle, and that endothelium-dependent relaxation is mediated by cyclic GMP. A decrease in ATP would affect the capacity to produce the second messenger cyclic nucleotide.

Cause of High-Energy Phosphate Reduction

The mechanism for the decrease in high-energy phosphate levels after SAH is not clear. Possible explanations include encasement of the artery and toxic effects of substances released from the clot. Metabolic impairment of the cerebral vasculature can take place as a result of encasement; the cerebral arteries lack vasa vasorum and possess pores providing communication between the adventitial surface and the smooth-muscle layer, which may function as a pathway for feeding and draining. A recent study reported that oxyhemoglobin, when applied to isolated smooth-muscle cells, causes contraction, membrane damage, an increase in outward potassium current, a decrease in membrane resistance, and finally cell death; by contrast, oxyhe-
moglobin did not have such an effect on neuroblastoma cells. If oxyhemoglobin exerts a damaging effect on the smooth-muscle cellular membrane, a decrease in high-energy phosphates can be secondary to increased permeability, or due to impaired metabolism as the result of general cell failure caused by the calcium overload.

A partial resolution of angiographic vasospasm was observed between Day 7 and Day 14 after SAH. In that period, the markers of metabolism did not recover. On Day 14, the arteries were still in metabolic derangement of the same or increased severity, but vasospasm was ameliorated. The reason for the discrepancy is not clear; it suggests that, after a period of prolonged energy-deficient status, the persisting impairment of the intracellular milieu no longer sustains the pathological constriction. This could possibly be due to damage to the integrity of the cells, resulting in leak or loss of protein components essential for maintenance of contraction.

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References


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