Metabolic Profiles of Canine Cerebrovascular Tree: A Histochemical Study

B. H. Cook, M.D., Ph.D., H. J. Granger, Ph.D., D. N. Granger, Ph.D., A. E. Taylor, Ph.D., and E. E. Smith, Ph.D.

SUMMARY Intriguing questions have recently been raised regarding the applicability of direct observations of the pial microcirculation to the behavior of the total cerebral microcirculation. Operating under the assumption that arteriolar tone and, thus, cerebrovascular resistance is, to some extent, directly related to the intrinsic energy metabolism of the arteriolar wall, a comparative histochemical analysis of cerebral microvessels, both pial and parenchymal, was undertaken. Reactions were chosen on the bases of representation of substrate and of enzymes of glycolysis, the hexose monophosphate shunt, \( \beta \)-oxidation of fat, Krebs cycle, cytochrome system and ATP hydrolysis. Three metabolically distinct segments of the cerebral microvasculature were delineated with the pial vessels showing strong capacities for glycolysis, \( \beta \)-oxidation of fats and utilization of glucose through the hexose monophosphate shunt. Microvessels of the gray matter have a qualitatively similar metabolic profile but the capacities of each pathway are lower when compared to pial arterioles. Arterioles of the white matter demonstrate the weakest energy-yielding capacities.

SINCE THE DIRECT observations of the pial circulation by Donders in the 19th century and by Forbes in the early 20th century, the responses of the pial vessels have served as a useful model for the whole brain microcirculatory unit. However, recently raised the intriguing question of whether the pial vessels do indeed mimic the responses of parenchymal vessels. While pial vessels have been shown to respond in a proper manner to those humoral substances (CO\(_2\), H\(^+\)) generally accepted to be responsible for cerebral autoregulation, other studies have indicated that parenchymal vessels behave in a manner which would tend to compensate for alterations in pial vascular calibre. In addition to neurogenic and parenchymal (via vasodilator release) factors, vascular tone is, to some extent, a function of the metabolic properties of the arteriolar wall which mediate the contractile process through production of adenosine triphosphate. Since previous studies in this laboratory have indicated that a metabolic heterogeneity between vessels of different calibre within a vascular bed and between vessels of similar calibre from organ to organ may exist, the tophohistochemical approach utilized in these studies has been applied to the cerebral microvasculature in order to ascertain whether the vessels of the pia are metabolically similar to parenchymal vessels.

Methods

Ten mongrel dogs were anesthetized with sodium pentobarbital. Skin and scalp muscles overlying the skull were dissected away before a rectangular opening was made in the fronto-parietal bones for biopsy of underlying cerebral cortex. Biopsied samples were immediately frozen at \(-170^\circ\)C in isopentane precooled in liquid nitrogen. Ten micron sections were cut on an Ames Lab-Tek cryostat and were not allowed to thaw prior to the application of incubating medium. Standard histochemical reactions were performed for representative enzymes of each of the major metabolic
"Nothing dehydrogenase" activity was eliminated through malin vapors for 5 minutes prior to incubation respectively. through exposure of the unfixed tissue section to hot formic acid omission of substrate from the incubation medium and cerebral vasculature.

Table 1

Reactions Employed for Histochemical Analyses

<table>
<thead>
<tr>
<th>A. Substrates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycogen</td>
<td>14</td>
</tr>
<tr>
<td>Neutral Fat</td>
<td>15</td>
</tr>
<tr>
<td>Free Fatty Acids</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Glycolysis lactate dehydrogenase</td>
<td>9</td>
</tr>
<tr>
<td>2) Hexose Monophosphate Shunt glucose-6-phosphate dehydrogenase</td>
<td>9</td>
</tr>
<tr>
<td>DPN diaphorase</td>
<td>18</td>
</tr>
<tr>
<td>3) Lipid Catabolism lipase</td>
<td>13</td>
</tr>
<tr>
<td>β-OH butyrate dehydrogenase</td>
<td>9</td>
</tr>
<tr>
<td>4) Tricarboxylic Acid Cycle NAD-linked isocitrate dehydrogenase</td>
<td>9</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>9</td>
</tr>
<tr>
<td>5) Respiratory Chain cytochrome oxidase</td>
<td>12</td>
</tr>
<tr>
<td>6) ATPases</td>
<td></td>
</tr>
<tr>
<td>Herman-Padykula ATPase</td>
<td>10</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>11</td>
</tr>
</tbody>
</table>

Controls for enzyme reaction were performed through standard methods of Hess, Pearse, and Scarpelli utilizing polyvinyl alcohol to minimize enzyme diffusion and phenazine methosulfate as an intermediate electron acceptor. Myosin ATPase was demonstrated by the calcium-cobalt method of Hermann and Padykula and alkaline phosphatase was demonstrated using the naphthol AS phosphate method of Burstone. Cytochrome oxidase localization was afforded by Burstone's amino-quinoline method. Lipase was demonstrated utilizing Gomori's Twen 80 method for "true lipase." The dimedone-PAS method of Bulmer was employed for glycogen demonstration.

In general, a histochemical profile was constructed for both the energy producing reactions (glycolysis, hexose-monophosphate shunt, β-oxidation of fats, and the respiratory chain) and energy yielding reactions (ATPases). In addition, cellular equipment for the breakdown of neutral fat to triglyceride was studied in conjunction with Lillie's Oil Red O method for neutral fat and Holzinger's method for free fatty acids. Table 1 summarizes the reactions performed for delineation of metabolic characteristics of cerebral vasculature.

Controls for enzyme reaction were performed through omission of substrate from the incubation medium and through exposure of the unfixed tissue section to hot formalin vapors for 5 minutes prior to incubation respectively. "Nothing dehydrogenase" activity was eliminated through rigid pH control. A 30 minute acetone extraction provided controls for the Oil Red O reaction.

For each histochemical reaction, color photographs of 5 to 6 microvessels were obtained from each brain compartment of each dog under identical magnification and illumination conditions. Hence, 50 to 60 photographs of pial vessels could be compared with an identical number of microvessels of grey or white matter. After random pairing of photographs representing two compartments, two individuals working separately ranked each pair according to intensity of specific histochemical color products located within the muscle layer of the microvessels. This process was repeated until all permutations were ranked (i.e. pia vs white matter, grey vs white matter, pia vs grey matter).

A difference in reaction intensity was considered highly probable if 80% of the paired photographs showed a consistent gradient of staining. We assumed that no difference existed if only 40 to 50% of the paired photos for a given reaction yielded positive rankings for an assumed gradient. Because all tissue sections were identical in thickness, the variations in intensity cannot be explained by differences in substrate penetrability unless the physical consistency of cytoplasm within microvascular smooth muscle varies from compartment to compartment, an unlikely possibility.

After the relative rankings of the 3 brain compartments were established, an attempt was made to provide a subjective index of the magnitude of the gradient existing between 2 compartments meeting our criteria for significant difference. This subjective index provides a crude means of determining whether these consistent gradients of histochemical activity are shallow or steep.

Pial vessels larger than 100 µ were excluded from this study due to an apparent heterogeneity which precluded application of our method of analysis.

Results

Table 2 illustrates the subjectively graded observations for vessels of the pial, grey matter, and white matter segments of the cerebral vasculature. Pial vessels of 15–75 µ diameter were observed in random distribution within the sulci of the cerebral cortex. No significant metabolic differentiation could be distinguished among these pial vessels. Surprisingly, however, our results indicate the parenchymal vessels cannot be compartmentalized as a single entity. On the contrary, we were able to histochemically delineate a difference between vessels of the grey and white matter. For reasons of coherence the results are categorized with reference first to the function of the molecule involved, i.e. substrate vs enzyme, and enzymes are further subdivided with reference to the metabolic pathway they represent.

1. Substrates

By referring to table 2, the reader will note the gradient demonstrated for glycogen with the highest levels being found in the pial vasculature. It appears that as the vessel penetrates the substance of the brain the glycogen of arteriolar smooth muscle decreases with intermediate amounts present in vessels of the grey matter and lesser amounts in those of the white matter.
Neutral fat, as demonstrated by Lillie's Oil Red O technique, was found to be weakly positive in the pial vasculature and essentially absent from the vessels of grey and white matter.

Free fatty acids were found to be present in moderate concentrations in the three segments of the vasculature.

II. Enzymes

A. Glycolysis. Lactate dehydrogenase was utilized as an indicator of glycolytic activity. Zugibe\textsuperscript{12} regards the activity of this enzyme as the "most useful of all the histochemical techniques to determine whether the Embden-Meyerhof pathway is operative." The activity of this enzyme was found to be greatest in the pial vessels with decreasing activity as the vessels traversed grey and finally white matter.

B. Hexose monophosphate shunt. The relative significance of this pathway was evaluated through the activity of both the initial enzyme of the pathway, glucose-6-phosphate dehydrogenase, and of the final mediator of ATP production from this pathway, DPN diaphorase. Cohen\textsuperscript{18} regards the latter enzyme as reflecting the activity of transhydrogenase. Maximal glucose-6-phosphate dehydrogenase activity was found in the superficial (pial) vessels of the brain with a decrease in the vessels of the grey matter and a further decrease in the vessels of the white matter. DPN diaphorase shows a similar pattern with greatest intensity in the surface vasculature and decreasing intensity in grey and white matter.

C. Tricarboxylic acid cycle. The oxidative capabilities of the tricarboxylic acid cycle of the three vascular segments were evaluated through the activities of NAD-linked isocitrate dehydrogenase and of succinate dehydrogenase. Isocitrate dehydrogenase was quite intense in all vascular segments studied with no differential observed. Succinate dehydrogenase, on the other hand, showed the greatest activity in pial vessels with low intensities in the vasculature of both grey and white matter.

D. Lipid metabolism. As noted before, the level of non-globular neutral fat, as tested by the Oil Red O technique, was very slight in the pial vasculature. No detectable non-globular neutral fat was present in vessels of grey and white matter. Moderate lipase activity was present in the surface vasculature of the brain with a total absence in both grey and white matter. \(\beta\)-OH butyrate dehydrogenase, utilized as an indicator of \(\beta\)-oxidation of fats, was found to be extremely active in pial vessels with lesser amounts in grey matter and a further decline in the vasculature of white matter. The presence of free fatty acids and TCA cycle in the pial vessels serve to reinforce the concept of a \(\beta\)-oxidative scheme in these vessels.

E. The respiratory chain. The activity of this enzyme reflects the terminus in the cycle of oxygen-dependent ATP production. Pial vessels demonstrated higher activity of this enzyme than the vessels of grey and white matter.

F. Enzymes of energy utilization. Myosin ATPase was noted to be extremely active in all three vascular segments studied. Alkaline phosphatase, on the other hand, was absent from the pial vasculature but present in high concentration in the arterioles of both grey and white matter.

Discussion

Controversy has existed for some time regarding the validity of direct observation of pial vasculature and extrapolation of this data to the microvasculature of the brain as a whole. While our results do not negate this approach, we have found intrinsic metabolic dissimilarities between not only pial and parenchymal microvessels but have, in addition, observed a further differentiation of microvascular metabolic capabilities between vessels of grey and those of white matter. In referring to table 2, it will be noted that pial vessels possess strong LDH activity, suggesting an active role of the Embden-Meyerhof pathway in the metabolism of these arteriolar circuits. The strong activity of hexose monophosphate shunt enzymes in conjunction with high glycogen levels suggests, on the other hand, that this pathway may play a role in the maintenance of arteriolar wall tone. Again, examination of this table with reference to the metabolic paraphernalia required for fat catabolism reveals a great potential for this conduit for ATP production. A slight reaction for non-globular neutral fat probably reflects small amounts of either constitutive or metabolizable liquid. In conjunction with this we find a moderate lipase activity and an intense reaction for free fatty acids. Quite striking is the presence, in quantity, of reaction products for the enzymes necessary to catabolize free fatty acid to CO\(_2\) and H\(_2\)O. This is reflected in the reactions for \(\beta\)-hydroxybutyrate dehydrogenase, NAD\(^+\) linked isocitrate dehydrogenase, succinate dehydrogenase, and cytochrome oxidase. In addition, the intense activities of Hermann-Padykula ("myosin") ATPase suggests a high capacity for utilization of generated adenosine triphosphate.
Microvessels of grey matter seem quite capable of utilizing the Embden-Meyerhof pathway as suggested by moderate lactate dehydrogenase activity. These vessels, although seemingly capable of utilization of oxidative pathways for both lipid and carbohydrate, seem somewhat less endowed than those of the pia. The absence of neutral fat and lipase together with the moderate reaction for free fatty acids suggests little intramural lipid storage with fatty acids of the arteriolar wall provided exclusively via delivery by the circulatory system. The importance of the hexose-monophosphate shunt, as judged by the intensity of its enzymes, would seem comparatively less than in pial vessels. “Myosin” ATPase was demonstrated to have an activity equal to that of the pial vessels. Interestingly, alkaline phosphatase, another of the ATPases, was found to be reactive in the arterioles of grey matter while totally absent in the pial vasculature.

Finally, the microvessels of white matter demonstrated, in general, markedly reduced levels for most of the reactions performed. In contrast to pial vasculature, this bed seems to lack the capacity for anaerobic glycolysis, as indicated by the absence of detectable levels of lactate dehydrogenase activity. Like the vessels of grey matter there seemed to be no endogenous mechanism for storage and initial (triglyceride → free fatty acid) catabolism of neutral fat. There seemed, in addition, to be a definite, yet once again greatly reduced, capacity for aerobic lipid metabolism. This may, however, be misleading in view of the intense activity of NAD⁺ linked isocitrate dehydrogenase, a rate limiting step in the tricarboxylic acid cycle. It is therefore plausible that the rate of oxidative metabolism in these vessels is greater than indicated by the relatively weak reactions for succinate dehydrogenase and cytochrome oxidase. The total carbohydrate metabolism of these vessels seems to be operative through the hexose monophosphate shunt and the aerobic component of the Embden-Meyerhof pathway. "Myosin" ATPase and alkaline phosphatase, as has been shown in the vessels of grey matter, were noted to be quite intense in white matter arterioles.

In summary, the superficial vessels of the canine cerebral cortex seem metabolically to be the most active vessels of the cerebral microcirculation with high capacities for glycolysis, Krebs cycle, lipid catabolism and the hexose monophosphate shunt. Arterioles of the grey matter have metabolic capabilities qualitatively similar but quantitatively reduced when compared to the pial microvasculature. Vessels of the white matter were demonstrated to possess, in the smallest quantities noted, the enzymes of glycolysis, β-oxidation, the tricarboxylic acid cycle, the hexose monophosphate shunt, and the respiratory chain. All vessels studied were found to be rich in myosin ATPase with only the arterioles of grey and white matter demonstrating alkaline phosphatase activity.

Thus, insofar as the vascular wall of the pial, grey matter and white matter circulation contribute directly to cerebrovascular resistances, these enzyme and substrate patterns assume some degree of importance. It is quite conceivable that changes in cerebrovascular resistance in response to altered pH, Pco₂, etc., could well be mediated through effects on enzyme systems of the arteriolar wall. If this assumption is tenable, then it is well to note the existence of the three metabolically distinct vascular segments described herein and to analyze their behavior in view of the metabolic pathways which they employ.

Acknowledgment

The authors are indebted to Teresa Stoddard and Sandy McCormick for secretarial assistance.

References

1. Donders FC: Die Bewinginen Der Hersenen en De Ver Veranderingen der Vanpelling Van de Pia Mater. Nederlanden Lances 521–553, 1849
14. Bulmer D: Dimedone as an aldehyde blocking reagent to facilitate the histochemical demonstration of glycogens. Stain Techn 34:95-98, 1959
15. Lillie RD: Various oil soluble dyes as fat stains in the supersaturated isopropanol technique. Stain Techn 19: 45, 1944
Metabolic profiles of canine cerebrovascular tree: a histochemical study.
B H Cook, H J Granger, D N Granger, A E Taylor and E E Smith

Stroke. 1978;9:165-168
doi: 10.1161/01.STR.9.2.165

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1978 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/9/2/165

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/