A Histochemical Study of Cerebral Cortical Vessels and Ganglionic Vessels of the Caudatoputamen in Aging Normotensive Rats

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SUMMARY The goal was to describe the metabolic profile of ganglionic and cortical arteries and arterioles in aging normotensive male rats. Five enzymes indicative of key metabolic pathways in the vessel walls were semiquantitatively evaluated using bright-field histochemical microscopy. Lactate dehydrogenase showed significant reactivity which increased with vessel diameter in cortical and ganglionic vessels in all age groups tested. Succinate dehydrogenase and cytochrome oxidase showed little reactivity in both cortical and ganglionic vessels, suggesting a reduced role for aerobic metabolic pathways. Myosin ATPase reactivity was high in cortical and ganglionic vessels. Only this enzyme showed an increased reactivity that was correlated with the age and diameter of the vessel. Glucose-6-phosphate dehydrogenase reactivity was more pronounced in cortical than ganglionic vessels, suggesting that the hexose-monophosphate-shunt may be more active in the cortical vessels. There were no regional differences in enzyme reactivity throughout the caudatoputamen. In conclusion, both the cortical and ganglionic vessels are metabolically active, with significant anaerobic glycolysis, and reduced, but observable capacity for aerobic metabolism. The decreased myosin ATPase reactivity and the low level of glucose-6-phosphate dehydrogenase reactivity in the ganglionic arterioles of senescent rats may contribute to the susceptibility of these vessels to cerebrovascular accidents.

Methods Twenty-three male rats (Charles River, COBS CD outbred albino) were placed into four age groups. The youngest rats utilized (3 months old) and the 10–12 month-old retired breeders were purchased from Charles River. Some of the retired breeders were maintained on standard rat chow and water ad libitum until 25–27 or 31–33 months old. The number of rats used in each age group was: 1) 3 months old = 9; 2) 10–12 months old = 4; 3) 25–27 months old = 6; and 4) 31–33 months old = 4.

The systolic pressure of each animal was obtained using a Narco-Biosystems indirect blood pressure measurement system. These pressures in all age groups ranged from 104–128 mm Hg and were within normotensive limits. Each animal was anesthetized with intraperitoneal injections of sodium pentobarbital (45 mg/kg body weight) and placed into a stereotaxic instrument. A coronal slab of brain containing the CPU and the cortical and ganglionic vessels was obtained as follows: the bone of the dorsum of the skull extending from the caudal part of the orbit to the suture-line bregma was removed to expose a 6–8 mm wide strip of dura mater. After smoothing the bony edges bordering the dural strip, a single-edged razor blade was passed through the brain at the caudal and rostral edges of the exposed strip. This coronal slab was removed and rapidly frozen in a mixture of isopentane cooled in an acetone-dry ice mixture. The tissue was kept in an ultracold freezer (−40°C) until utilized for the histochemical determinations.

Histochemical Techniques The frozen samples were mounted on cryostat chucks and sectioned at 6 μm on an American Optical microtome-cryostat at −15°C. Using these sections, five enzymes representative of key metabolic pathways in the arterial and arteriolar walls were histo-
TABLE 1 Five Enzymes Evaluated in the Histochemical Analyses of the Metabolic Profiles of Cortical and Ganglionic Vessels

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>Anaerobic glycolytic capacity (Embden-Myerhof Pathway)</td>
</tr>
<tr>
<td>Succinate dehydrogenase (SDH)</td>
<td>Aerobic metabolism (Kreb's cycle)</td>
</tr>
<tr>
<td>Cytochrome oxidase (CyChrOx)</td>
<td>Aerobic metabolism (Oxidative phosphorylation and respiratory chain)</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (G-6-PDH)</td>
<td>Hexose-monophosphate-shunt activity</td>
</tr>
<tr>
<td>Myosin ATPase (MyATPase)</td>
<td>Indicator of ATP utilization (contraction of smooth muscle)</td>
</tr>
</tbody>
</table>

Abbreviations:

ACA    anterior cerebral artery
CC    corpus callosum
CI    internal capsule
CPU    caudatoputamen
CyChrOx    cytochrome oxidase
FX    fornix
GP    globus pallidus
L    lumen
LDH    lactate dehydrogenase
MCA    middle cerebral artery
MyATPase    myosin ATPase
PCA    posterior cerebral artery
SDH    succinate dehydrogenase
TOL    lateral olfactory tract
v    ventricle

chemically examined. These enzymes and their known functions are listed in table 1. Succinate dehydrogenase (SDH) and cytochrome oxidase (CyChrOx) were assessed to evaluate the aerobic metabolic capacity (Kreb's cycle, oxidative phosphorylation and respiratory chain). For anaerobic glycolytic capacity, the enzyme lactate dehydrogenase (LDH) was examined. To evaluate the capacity of the hexose-monophosphate-shunt, the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) was chosen. Myosin ATPase (MyATPase) activity was appraised as an index of the ability to break high-energy phosphate bonds and to utilize ATP for smooth muscle contraction.

The dehydrogenases were demonstrated using the substrate solutions as outlined by Pearse\textsuperscript{16} with nitro BT as the tetrazolium salt and incubated for 60 minutes at 37°C. The cytochrome oxidase procedure of Burstone\textsuperscript{17} was employed using p-aminodiphenylamine and naphthol AS-L3G with and without the addition of cytochrome C to the substrate. Incubation was carried out for 3 hours at room temperature followed by transfer of the sections to 1% cobalt acetate in 10% formalin for 1 hour. The Padykula-Herman method as modified by Pearse\textsuperscript{16} was used for myosin ATPase, with the tissue sections incubated for 25 minutes at 37°C. Because of the extreme intensity of this reaction in the arteriolar walls, the concentration of ATP in the final incubation medium was reduced to 0.36 mM. Negative controls for each of the enzymatic reactions consisted of 1) deletion of specific substrate and 2) exposure of the cold tissue sections to boiling formalin vapor for 10 minutes, or where appropriate, 3) the addition of sodium azide (2M) or potassium cyanide (2M) to the final incubation medium as a respiratory inhibitor. Positive controls consisted of tissues known to exhibit reactivity. Succinate and lactate dehydrogenase and cytochrome oxidase were demonstrated in rat liver, kidney and cardiac muscle, while glucose-6-phosphate dehydrogenase was shown in liver and kidney. Myosin ATPase was demonstrated in rat cardiac and skeletal muscle and in smooth muscle of portions of the small intestine.

Sampling Procedures for Cortical and Ganglionic Vessels

The outside diameters (od in micrometers) were measured in 1,811 vessels (435 cortical; 1,376 ganglionic) using an ocular micrometer. Many vessels were cut in nearly circular profile since the tissue sections examined were 6 μm thick. In noncircular profiles of vessels, the recorded diameter was the mean diameter determined by summing the length of the long and short axes and dividing by two. When a vascular profile was a long sagittal section with distinct lumen and approximately equal wall thicknesses, as occurred with some ganglionic vessels, the outside diameter (od) was determined by measuring the width of the vessel. Any vessel cut in a plane so that the lumen was not visualized was excluded.

Cortical vessels were identified as part of the anterior (ACA), middle (MCA) or posterior cerebral (PCA) arteries or the circle of Willis based upon their relative positions respective to structures within the coronal brain sections. Ganglionic vessels supplying the CPU were identified by their size (90% had od ≤70 μm); moreover, the CPU has discrete recognizable boundaries in unstained tissue sections (fig. 1). The CPU was subdivided into rostral (A:10–7.6 mm), middle (A:7.6–6.4 mm), and caudal (A:6.4–4.6 mm) portions utilizing the stereotaxic atlas of Pelligrino and Cush-
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These subdivisions were done to determine if the metabolic profiles of the vessels supplying these three regions were as dissimilar as the previously-described variations in patterns of blood supply to these portions of the CPU. All tissue sections were examined simultaneously by the same two observers using bright-field microscopy. Vessels were photographed at the same light intensity and magnification, then compared for staining intensity. The reactivity of each enzyme was semi-quantitatively estimated on a scale of 0 to +4, where: 0 = no discernible reaction, and ± = trace; +1 = slight; +2 = moderate; +3 = strong; and +4 = very strong reactions, respectively. In addition, some sections were stained with 2% aqueous methyl green or cresyl violet and examined for tissue orientation.

Electron Microscopy

Transmission electron microscopy was used to examine small arterioles (od ≈ 10 μm) in the CPU of four rats 20–25 months old. In particular, we attempted to discern if smooth muscle occurred in the walls of these small vessels. Each animal was anesthetized, and infused through the heart with a 0.1 M sucrose-phosphate buffer followed by a solution of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M sucrose-phosphate buffer. Coronal slabs containing the CPU were excised, trimmed, cut into 1 mm³ blocks, and post-fixed in cold 1% osmium tetroxide in sucrose-phosphate buffer for 2 hours at 2–5°C. The blocks were dehydrated and embedded in epoxy resin. Thick sections were cut and stained with toluidine blue and examined with a Hitachi 500 electron microscope.

Results

The relative amounts of five enzymes found in rat cortical and ganglionic cerebral vasculature are demonstrated graphically. Four age groups of normotensive rats are examined; specifically, 3; 10–12; 25–27 and 31–33 month-old animals. Two graphs are presented for each enzyme, one for cortical and one for ganglionic vessels, respectively (figs. 2A,B; 4A,B; 7A,B; 8A,B; 9A,B).

Myosin ATPase

Myosin ATPase in cortical vessels demonstrated slight to strong activity (fig. 2A). The most uniform reactivity occurred in the 10–12 month-old animals, where most vessels, regardless of diameter, showed moderate activity. In contrast, the ganglionic vessels exhibited three distinct age-related patterns for myosin ATPase reactivity. The 3 month and nearly all the 10–12 month-old rats showed moderate activity, while the 25–27 month-old animals demonstrated a strong to very strong response. The 31–33 month-old rat ganglionic vessels were the least reactive (fig. 2B). There were no differences in reactivities in ganglionic vessels between distinct areas of the caudatoputamen. In all cortical and ganglionic vasculature, regardless of diameter, enzyme activity was demonstrated within the vessel wall (fig. 3A–C). Ultrastructural examination of small (4–9 μm) ganglionic vessels in 20–25 month-old rats revealed smooth muscle cells surrounded by basal laminae (fig. 3D). Utilizing bright-field histochemistry, myosin ATPase was demonstrated in the smooth muscle. However, in the smaller vessels it was difficult to distinguish the endothelial cells from the adjacent smooth muscle, and therefore whether the endothelial cells contained reaction product for myosin ATPase.

Anaerobic Glycolysis

Lactate dehydrogenase in cortical vessels exhibited moderate to strong reactivity for all age animals (fig. 4A), with the exception of the largest cortical vessels in the 3 month-old rats, which demonstrated very strong activity (fig. 5A–D). In ganglionic vessels, lactate dehydrogenase reactivity paralleled increase in vascular size in all age animals (figs. 4B,6A–C) and was similar throughout the caudatoputamen.

Aerobic Metabolism

All cortical vessels showed slight to moderate reactivity for succinate dehydrogenase in all age rats (fig. 7A). In virtually all ganglionic vessels, regardless of

Figure 2A,B. Graphic representation of myosin ATPase (MyATPase) reactivity with increasing vessel diameter for cortical (A) and ganglionic (B) vasculature is shown. Enzyme reactivity from four age groups of rats ranging from 3 months to 31–33 month-old animals is plotted. Intensity of reaction: 0 = no discernible reaction; ± = trace; +1 = slight; +2 = moderate; +3 = strong; and +4 = very strong reactivity, respectively.
animal age, succinate dehydrogenase exhibited trace to slight activity; the only exception occurred in the smallest vessels of the oldest rats where no enzyme reactivity was detected (fig. 7B).

In cortical vessels, cytochrome oxidase activity ranged from zero to slight for all age rats (fig. 8A). There was no uniform pattern between reactivity, vessel diameter, and age. Ganglionic vessels generally demonstrated zero to trace activity for cytochrome oxi-

In cortical vessels, cytochrome oxidase activity ranged from zero to slight for all age rats (fig. 8A). There was no uniform pattern between reactivity, vessel diameter, and age. Ganglionic vessels generally demonstrated zero to trace activity for cytochrome oxidase, excepting the smallest vessels in the 10–12 month-old animals which showed slight reactivity (fig. 8B). The reactivities for each enzyme, respectively, in ganglionic vessels, showed no regional variations throughout the caudatoputamen.

Hexose-monophosphate-shunt

In the cortical vessels of all age animals, glucose-6-phosphate dehydrogenase showed trace to moderate activity (fig. 9A). Furthermore, in the four age groups, this enzyme increased in reactivity as the cortical vessels increased in size. The ganglionic vessels showed zero to trace reactivity for glucose-6-phosphate dehydrogenase, with the exception of the 4–9 μm vessels in the 10–12 month-old animals which demonstrated slight activity (fig. 9B). The reactivities in ganglionic vessels showed no regional variability throughout the caudatoputamen.

Discussion

The metabolic profile of each enzyme examined in the rat cortical and ganglionic vessels shows only minor differences as the vessels age, with the exception of myosin ATPase in ganglionic vessels. Furthermore, the degree of reactivity for specific enzymes falls into narrow ranges and shows a clear correlation with vessel diameter only in the case of lactate dehydrogenase in ganglionic vessels. There also is no noticeable difference in reactivity for specific enzymes in ganglionic vessels supplying different areas of the caudatoputamen, in contrast to the distinctly different morphological patterns of these vessels. This apparent uniformity of specific enzyme reactivity throughout the caudatoputamen suggests that the increased incidence of stroke in the microvasculature of the rostral parts of the caudatoputamen (head and body) is more closely correlated with the morphology (small diameter, serpentine) of these vessels.

Regarding myosin ATPase in ganglionic vessels, its
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FIGURE 4A,B. Graphic representation of lactate dehydrogenase (LDH) reactivity with increasing vessel diameter for cortical (A) and ganglionic (B) vasculature is shown. Enzyme reactivity from four age groups of rats ranging from 3 months to 31-33 month-old animals is plotted. Intensity of reaction: 0 = no discernible reaction; ± = trace; +1 = slight; +2 = moderate; +3 = strong; and +4 = very strong reactivity, respectively.

presence in the smooth muscle of even the smallest vessels strongly suggests utilization of generated adenosine triphosphate for muscular contraction. The generally high reactivity of lactate dehydrogenase, the enzyme catalyzing the interconversion of lactic and pyruvic acid, suggests that anaerobic glycolysis is a metabolically significant path in both cortical and ganglionic vessels. Succinate dehydrogenase and cytochrome oxidase, enzymes fundamental to aerobic metabolism, show slightly more reactivity in cortical than ganglionic vessels.

Ganglionic vasculature is more subject to stroke...
than the generally larger cortical vessels,21, 22 and the mechanisms of stroke are thought to be similar in man and the rat.1 The marked reduction in internal diameters at branch points, the acute angles of departure from larger parental cortical vessels and the serpentine course of ganglionic vessels are structural features that increase the susceptibility of these vessels to hemodynamic stresses.3 The present study suggests several metabolic factors which also may contribute to the stroke-prone susceptibility of the ganglionic vasculature. In general, the presence of significantly lower levels of myosin ATPase in the muscular walls of older ganglionic arterioles may render the walls of these vessels less responsive to physiologic and hemodynamic stimuli15, 17 and perhaps simply less pliable in adjusting to mechanical distortion.

Proliferative changes in the intima and media of cerebral vessels are a normal occurrence in aging human and rodent cerebral vasculature. Kojimahara and co-workers22 reported increases in elastin and collagen in the media, and an increase in basement membrane-like substances in larger cerebral vessels of rats 10-12 months of age and older. Commercially available rats live approximately 36 months23 and a 10-12 month-old rat chronologically approximates the third to fourth decade in man. Aging human cerebral vessels at this time show proliferation of the internal elastic membrane, intimal cellular thickening and increased numbers of smooth muscle cells.11, 12 The significance of these proliferative changes is uncertain, but they may provide a means of maintaining vessel integrity. Maintenance of vessel integrity may also be a function of the hexose-monophosphate-shunt, the sole cellular source of ribonucleotides necessary for the synthesis of new cellular protein.24 The observed increase in activity in the shunt may be correlated with the proliferative changes reported for aging cortical vessels.11, 12, 22 This shunt also may function in the production of intermediates for anaerobic glycolysis.25 Glucose-6-phosphate dehydrogenase catalyzes the initial reaction of the hexose-monophosphate-shunt. In the present study this enzyme generally demonstrates substantially less reactivity in ganglionic than cortical vessels. It may be that these ganglionic vessels possess little capacity for proliferative repair when weakened and torn, particularly with development of hypertension.1, 3

Our observation of high levels of lactate dehydrogenase in rat cortical vessels is in agreement with Cook and colleagues,4 who observed strong reactivity for this enzyme in cortical vessels in canine cerebral cortex. In contrast to Cook and co-workers,4 our observations have encompassed vessels ranging from muscu-

Figure 6A–C. Lactate dehydrogenase activity in three progressively smaller ganglionic vessels from a 10-12 month-old normotensive male rat. An increase in enzyme activity occurs as vessels increase in diameter for all four age groups of animals. Calibration bar = 10 μm.
...lar arteries to precapillary arterioles that supply the cortex and subcortical structures in rats at four distinct ages. Thus, in the rat, cortical vessels of all sizes from young to old animals show consistently high levels of lactate dehydrogenase. In the rat ganglionic vasculature, regardless of animal age, increase in lactate dehydrogenase activity parallels increase in vessel diameter. Zugibe,26 regards lactate dehydrogenase activity as the most useful of histochemical techniques to determine anaerobic glycolytic reactivity via the Embden-Meyerhof pathway. It may well be that in the rat, the smaller ganglionic microvessels are significantly less dependent upon anaerobic metabolism than cortical vessels of comparable size.

As previously stated, succinate dehydrogenase and cytochrome oxidase, enzymes of aerobic metabolism, show slightly more activity in cortical than ganglionic vessels with little correlation to vascular size or animal age. Cytochrome oxidase never demonstrates more than slight reactivity in any size cortical or ganglionic vessel, while succinate dehydrogenase never shows greater than moderate activity, and then only in some medium-size cortical vessels in animals not exceeding 12 months of age. The low levels of cytochrome oxidase and succinate dehydrogenase suggest that aerobic pathways may play a lesser role in vessel metabolism. In fact, the low levels of cytochrome oxidase probably serve to inhibit use of O2 in aerobic metabolism.25

Histochemical techniques cannot always provide precise quantitation of enzyme activities, especially under the physiological and biochemical conditions existing in vivo. Rather, these techniques optimize the conditions of a biochemical reaction, thereby allowing appraisal of differences in the maximum capacity of respective enzymes in tissues too small for biochemical analyses or which cannot be isolated.25-29 In addition, the system of cortical and ganglionic vessels which we are presently examining is an extremely complex vascular bed; it is particularly difficult to approach, and to isolate individual cerebral tissues and vascular components. All these technical limitations are doubtless reflected in the relatively small number of studies concerning the metabolism of the cerebral vascular bed.4, 6, 7, 30

Thus, recognizing these limitations, in addition to the advantages which histochemical methodology affords, care must be taken in drawing conclusions regarding cortical and ganglionic vascular metabolism. In summary, both the cerebral vessels supplying the neocortex and ganglionic vessels throughout the rat caudatoputamen are metabolically active, and appear to depend predominantly upon anaerobic glycolysis. In addition, the low levels of myosin ATPase and glucose-6-phosphate dehydrogenase seen in the ganglionic microvasculature of older rats, may more readily dispose these vessels to cerebrovascular accident.
**Figure 9A, B.** Graphic representation of glucose-6-phosphate dehydrogenase (G-6-PDH) reactivity with increasing vessel diameter for cortical (A) and ganglionic (B) vasculature is shown. Enzyme reactivity from four age groups of rats ranging from 3 months to 31 to 33 months-old animals is plotted. Intensity of reaction: 0 = no discernible reaction; ± = trace; +1 = slight; +2 = moderate; +3 = strong; and +4 = very strong reactivity, respectively.

### References

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Stroke. 1985;16:285-292
doi: 10.1161/01.STR.16.2.285

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