# Hydroxyl Radical Production in the Cortex and Striatum in a Rat Model of Focal Cerebral Ischemia

*Line Ste-Marie, Pascal Vachon, Luc Vachon, Chantal Bémeur, Marie-Claude Guertin and Jane Montgomery*

**ABSTRACT:** ***Background:*** Increases in hydroxyl radical production have been used as evidence of oxidative stress in cerebral ischemia/ reperfusion. Ischemia can also induce increased dopamine release from the striatum that may contribute to hydroxyl radical formation. We have compared hydroxyl radical production in the cortex and striatum as an index of oxidative stress in a rat model of focal cerebral ischemia with cortical infarction. ***Methods:*** Using a three vessel occlusion model of focal cerebral ischemia combined with bilateral microdialysis, hydroxylation of 4-hydroxybenzoate (4HB) was continuously monitored in both hemispheres in either the lateral striatum or frontoparietal cortex. The ischemia protocol consisted of one hour equilibration, 30 min of three vessel occlusion, then release of the contralateral common carotid artery (CCA) for 2.5 h. ***Results:*** Induction of ischemia resulted in a 30-fold increase in dopamine release in the lateral striatum. Compared to the nonischemic striatum, the ratio of the hydroxylation product 3,4-dihydroxybenzoate (34DHB) to 4HB (trapping agent) in the ipsilateral striatum increased significantly 30 min after ischemia induction. In contrast, during the 30 min of three vessel occlusion there was no increase in the ratio in the cortex. Following the release of the contralateral CCA, the ratio from the ischemic cortex increased significantly compared to sham-operated animals. However, under all circumstances, the 34DHB/4HB ratio was greater in the striatum than in the cortex. ***Conclusion:*** The increase in the 34DHB/4HB ratio in the lateral striatum coincides with the increased dopamine release suggesting a role for dopamine oxidation in the increased production of hydroxyl radicals. The significant increase in the ratio from the ischemic cortex compared to that from the sham-operated animals is consistent with increased oxidative stress induced by ischemia. However, the lower 34DHB/4HB ratio in the cortex which does not receive dopaminergic innervation compared to the striatum suggests a different mechanism for hydroxyl radical production. Such an alternate mechanism may represent a more toxic oxidative insult that contributes to infarction.

**RÉSUMÉ: La production de radicaux hydroxyl dans le cortex et le striatum dans un modèle d'ischémie cérébrale focale chez le rat.** ***Introduction:*** Une augmentation de la production de radicaux hydroxyl a été utilisée comme évidence de stress oxydatif dans l'ischémie/la reperfusion cérébrale. L'ischémie peut également induire une augmentation de la libération de la dopamine du striatum qui peut contribuer à la formation de radicaux hydroxyl. Nous avons comparé la production de radicaux hydroxyl dans le cortex et le striatum comme index du stress oxydatif dans un modèle d'ischémie cérébrale focale avec infarcissement cortical chez le rat. ***Méthodes:*** Dans un modèle d'ischémie cérébrale focale impliquant l'occlusion de trois vaisseaux combinée à une microdialyse bilatérale, l'hydroxylation du 4-hydroxybenzoate (4HB) a fait l'objet d'une surveillance continue dans les deux hémisphères soit dans le striatum latéral ou le cortex frontopariétal. Le protocole d'ischémie comprenait une heure d'équilibration, 30 minutes d'occlusion de trois vaisseaux, puis la reperfusion de la carotide primitive contralatérale (CPC) pendant 2.5 heures. ***Résultats:*** L'induction de l'ischémie a augmenté de 30 fois la libération de la dopamine dans le striatum latéral. Comparé au striatum non ischémique, le rapport du produit de l'hydroxylation 3,4-dihydroxybenzoate (34DHB) au 4HB (agent capteur) dans le striatum ipsilatéral a augmenté significativement 30 minutes après l'induction de l'ischémie. Par contre, pendant les 30 minutes de l'occlusion de trois vaisseaux, il n'y avait pas d'augmentation du rapport dans le cortex. Suite à la reperfusion de la CPC, le rapport dans le cortex ischémique a augmenté significativement comparé à celui des animaux qui ont subi une opération factice. Cependant, dans toutes les circonstances, le rapport 34DHB/4HB était plus élevé dans le striatum que dans le cortex. ***Conclusions:*** L'augmentation du rapport 34FHB/4HB dans le striatum latéral coïncide avec l'augmentation de la libération de la dopamine suggérant un rôle de l'oxydation de la dopamine dans l'augmentation de la production des radicaux hydroxyl. L'augmentation significative du rapport dans le cortex ischémique comparé à celui d'animaux ayant subi une opération factice est compatible avec une augmentation du stress oxydatif induit par l'ischémie. Cependant, le rapport 34DHB/4HB plus bas dans le cortex qui ne reçoit pas d'innervation dopaminergique comparé au striatum suggère un mécanisme différent pour la production des radicaux hydroxyl. Un tel mécanisme pourrait impliquer une aggression oxydative plus toxique contribuant à l'infarctus.

Can. J. Neurol. Sci. 2000; 27: 152-159

Cerebral ischemia results in a cascade of events leading to a number of important cellular changes. These include rapid decreases in ATP, $Ca^{2+}$ release from intracellular stores, disruption of various ion pumps, excitotoxic changes resulting from glutamate release, acidosis and edema.[1-3] Many of these

From the Centre de Recherche du CHUM-Hôpital Notre-Dame and Département de Nutrition, Université de Montréal (L.Ste-M., L.V., C.B., J.M), Phoenix International, St. Laurent, Québec (P.V.) and the Centre de Recherche du CHUM-Hôpital Hôtel Dieu (M-C.G.)
RECEIVED JULY 13, 1999. ACCEPTED IN FINAL FORM FEBRUARY 16, 2000.
*Reprint requests to:* Jane Montgomery, Laboratoire de neurobiologie, CHUM-Hôpital Notre-Dame, M.8208, 1560 Sherbrooke St.E., Montréal, Québec, Canada H2L 4M1

changes are associated with increased free radical production which can occur both during ischemia and at reperfusion.

Middle cerebral artery (MCA) occlusion is one of the main causes of stroke observed in clinical settings. Reperfusion of the occluded artery is necessary to salvage the compromised ischemic tissue. Paradoxically, re-introduction of oxygen-rich blood to an ischemic tissue can result in reperfusion injury. It is suspected that reactive oxygen and nitrogen species (RONS) formed prior to or during reperfusion contribute to the tissue damage. A better understanding of the timing and factors that influence RONS formation is required for effective antioxidant intervention.

While the role of RONS in neuronal damage observed following focal cerebral ischemia with, or without, reperfusion remains unclear, there is substantial evidence for coincident increased production of RONS[4-10] and decreased endogenous antioxidants.[11,12] There are also reports of reduced damage when interceptive strategies are employed in models of cerebral ischemia.[8,13-18] One of the most reactive species is the hydroxyl radical and its production has been examined in a number of studies of focal ischemia[4-9] with the general consensus being that its production increases at reperfusion but may or may not during the intra-ischemic period.

Certain brain regions, such as the nigro-striatal pathway and the hippocampus, are thought to be more vulnerable to free radical damage. Striatal ischemia can be refractory to treatment compared to cortical ischemia, possibly because the nigro-striatal pathway appears to be susceptible to increased oxidative stress.[19,20] This susceptibility has been associated with dopamine oxidation (either enzymatic or nonenzymatic) that increases the production of hydrogen peroxide which, in the presence of iron ions, can decompose to produce hydroxyl radicals. The resulting highly reactive hydroxyl radicals can inflict damage to various cellular components. A number of groups have demonstrated that prior depletion of dopamine is neuroprotective in cerebral ischemia[21,22] suggesting that ischemia-induced dopamine release may contribute to increased oxidative stress.

In the present microdialysis study, we have characterized a three vessel (distal MCA and bilateral common carotid artery (CCA)) occlusion model of cerebral ischemia which results in cortical infarction. Partial reperfusion to the cortex was induced by reversing the contralateral CCA occlusion and was confirmed by laser-Doppler flowmetry. We have used bilateral microdialysis in the lateral striatum or the frontoparietal cortex to evaluate hydroxyl radical production. We have also quantitated dopamine release as an index of neurochemical changes in the lateral striatum induced by ischemia. We used the 4-hydroxybenzoate (4HB) method to trap hydroxyl radicals as 3,4-dihydroxybenzoate (34DHB).[23,24] Under all circumstances, hydroxyl radical production was higher in the striatum than in the cortex. As it is, the cortex and not the striatum that becomes infarcted in this model, the absolute amount of hydroxyl radicals that are produced during ischemia and early partial reperfusion does not appear to be related to the ultimate tissue damage that is observed. Conversely, it is possible that hydroxyl radical formation in the ischemic cortex involves a different mechanism than that in the striatum and that apparently smaller absolute amounts of hydroxyl radicals represent a more neurotoxic insult.

## Materials and Methods

***Materials:*** All chemicals were obtained from Sigma Chemical Co. High performance liquid chromatography (HPLC)-grade methanol and other solvents were used without further purification. Aqueous solutions were prepared using Milli-Q purified water and were filtered through 0.22 micron nylon filters prior to use.

***Microdialysis Probe Placement:*** All animal protocols were approved by the CHUM Research Centre's Animal Protection Committee to ensure compliance with guidelines established by the Canadian Council for Animal Care. A total of 16 male Sprague-Dawley rats (Charles River, Montréal, Québec) weighing 240-470 g were anesthetized by intra-peritoneal (ip) injection of chloral hydrate (400 mg/kg; maintenance dose, 20 mg every 40 min). Body temperature was kept at 37°C using a homeothermic blanket controlled by a rectal thermocouple (Harvard Apparatus). 4HB, the hydroxyl radical trapping agent,[23,25] was injected ip (400 mg/kg) at the beginning of the microdialysis equilibration period. Two microdialysis probes (CMA 12, 4 mm, CSC, Ville St-Laurent, Québec) were implanted bilaterally in either the lateral striatum (0 mm with respect to bregma, 3.8 mm lateral from the midline and 4.5 mm from the dura (n=6)) or the frontoparietal cortex (0 mm with respect to bregma, 4.2 mm lateral and 3 mm from the dura (n=6)).[26] The probes were perfused with Ringer's solution (pH 7) at 2 μl/min and microdialysis samples were collected into perchloric acid:EDTA (0.1 N:0.1%) at 10 min intervals during ischemia and at 15 min intervals at other times and were kept at 4°C prior to same day analysis.

***Measurement of Cerebral Blood Flow and Brain Temperature:*** Cortical cerebral blood flow (CBF) was measured under constant lighting conditions using laser-Doppler flowmetry (Transonic Systems). The needle probe was placed just above the dura, 2 mm behind the right microdialysis probe, after ensuring that there were no visible blood vessels. Brain temperature was monitored using a 21 guage needle thermocouple that was placed in the cortex lateral to the right microdialysis probe.

***Cerebral Ischemia Induction:*** A three vessel occlusion model similar to that described by Chen[27] and Buchan[28] was used to induce focal ischemia. Briefly, both common carotid arteries (CCAs) were isolated from the adjacent vagal nerves and were loosely encircled with 4-0 silk sutures drawn through PE-50 snares. Once the rat was positioned on the stereotaxic frame, a 3-4 mm temporal craniectomy was opened in line with bregma to visualize the right middle cerebral artery (MCA). After a one hour equilibration period following microdialysis probe insertion, ischemia was induced by cauterizing the distal MCA at the level of the inferior vein and quickly tightening the snares around both CCAs. Following 30 min of three vessel occlusion, the snare occluding the contralateral CCA was released and the microdialysis was continued for a further 2.5 hours. At this time, the right CCA occlusion was visually verified and then released and the neck and scalp incisions were sutured. Sham animals (n=2 for cortex and n=2 for striatum) were prepared as described except that the CCAs and right MCA were manipulated but not occluded. Microdialysis probes were positioned bilaterally in the cortex or the striatum prior to the sham procedure.



https://doi.org/10.1017/S0317167100052276 Published online by Cambridge University Press

***2,3,5-Triphenyltetrazolium chloride (TTC) staining:*** Following a 24 hour recovery period during which pain was controlled with buprenorphine (0.025 mg/kg subcutaneous injection bid), the rat was reanesthetized prior to sacrifice. Following rapid isolation of the brain, 2 mm coronal sections were cut and placed in a 2% TTC solution in 0.9% saline at 37°C for 30 min as described by Bederson.[29] The sections were then fixed in formaldehyde prior to photography. Ischemic areas were quantitated by planimetry which was validated in preliminary experiments by dissecting the ischemic zone from each slice and weighing the ischemic and nonischemic tissue and comparing the results with those obtained by planimetry.

***Hydroxyl Radical Trapping:*** We have developed a method analogous to the salicylate assay[30,31] using 4HB which, when hydroxylated, forms 34DHB. 4HB is given systemically to eliminate the relatively common problem of nonspecific hydroxylation induced by the microdialysis system.[23] Using systemic administration of salicylate, Althaus et al[16] have demonstrated that the production of 2,3- and 2,5-dihydroxybenzoate is proportional to the concentration of salicylate and variations in its concentration should be normalized using the ratio of the product to substrate. In the present study the analogous ratio is the concentration of the hydroxylation product, 34DHB to that of 4HB, the trapping agent.

***HPLC with Electrochemical Detection:*** 34DHB, 4HB, dopamine, DOPAC, HVA, norepinephrine and 5-HIAA were analyzed in microdialysis samples by HPLC (Waters 600-MS, Waters, Mississauga, Canada) with coulometric electrochemical detection (Coulochem II, Model 5011, SPE Ltd, Concord, Canada) as previously described.[23] The column used was a 2 mm x 150 mm Novapak $C_{18}$ column (Waters). The mobile phase was; 20 mM citric acid, 50 mM sodium acetate, 1.875 mM heptanesulfonate and 0.125 mM EDTA. The pH was adjusted to 4.05 with glacial acetic acid and methanol was added in the volume proportion of 93:7, buffer:methanol.

***Statistical analysis:*** Prior to analysis, a logarithmic transformation was applied to normalize the variances. The transformed data were analyzed using a three-way repeated measures analysis of variance (ANOVA), except for dopamine where a two-way repeated measures ANOVA was performed. Between factors were Region (cortex or striatum) and, for the ratio 34DHB/4HB, Group (ischemic hemisphere or sham-operated). Within factors were Time and Hemisphere. Based on the model used, p-values were calculated for all main effects and all interaction terms. In cases of a significant three-level interaction involving Region, subanalyses were performed separately for cortex and striatum using the appropriate mean sum of squares of the global model as denominator for the F ratios. Huynh-Feldt correction was applied to calculate the p-values associated with the within factors. For sham-operated animals, the data from both hemispheres were considered to be independent since our experience indicates that intra-animal variation is as great as inter-animal variation when bilateral microdialysis is performed.[32] All data are presented as the mean ± SEM. A p-value of 0.05 or less was considered significant. All analyses were performed with the SAS System (Release 6.12, Cary, NC, USA).

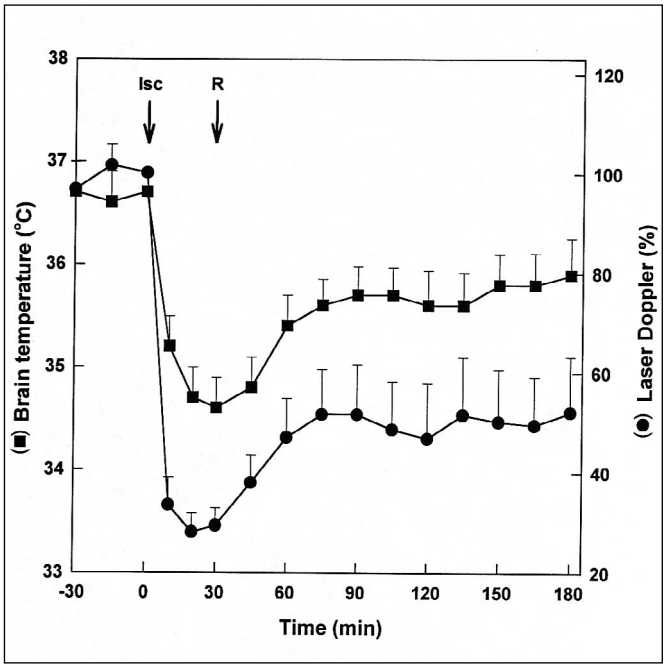


***Figure 1:*** *Cerebral Blood Flow Measured by Laser-Doppler and Brain Temperature during MCA Occlusion: Laser-Doppler readings (●) are expressed as a percent of their baseline values. Temperature changes (■) are presented in degrees Celsius. The error bars represent the mean ± SEM for 12 experiments.*

## RESULTS

***Cerebral blood flow, brain temperature and infarct area:*** Changes in cerebral blood flow, brain temperature and infarct area were used to evaluate the reproducibility of the three vessel occlusion model. Relative changes in cerebral blood flow (CBF) in the cortex as estimated by *in vivo* laser-Doppler flowmetry are presented in Figure 1. The results are expressed as a percent of baseline measurements to normalize for variable basal values. At the beginning of the three vessel occlusion, blood flow consistently decreased to 28 ± 4% of baseline levels and remained low during the 30 min of ischemia. Release of the contralateral CCA resulted in a partial reperfusion of the ischemic cortex to values that were 50 ± 10% below baseline. Release of the ipsilateral CCA resulted in a further increase in local CBF to approximately 75% of basal values (data not shown). In a similar model, Chen et al[27] observed decreases of 18 ± 4%, 48 ± 10% and 62 ± 11% during the three, two vessel and MCA-only occlusion periods, respectively. As also shown in Figure 1, the temperature of the ischemic cortex closely followed the change in laser-Doppler measurements. On average, brain temperature decreased by 2.2 ± 0.1°C during the 30 min ischemic period. Twenty-four hours after permanent distal MCA occlusion, we observed large cortical infarcts in coronal sections stained with TTC. At the level of bregma where the microdialysis probes were inserted, the infarct area corresponded to 35 ± 2% (n=12) of the affected hemisphere. We validated the TTC method by comparing our previous results with the infarct areas observed with cresyl violet staining following perfusion



https://doi.org/10.1017/S0317167100052276 Published online by Cambridge University Press

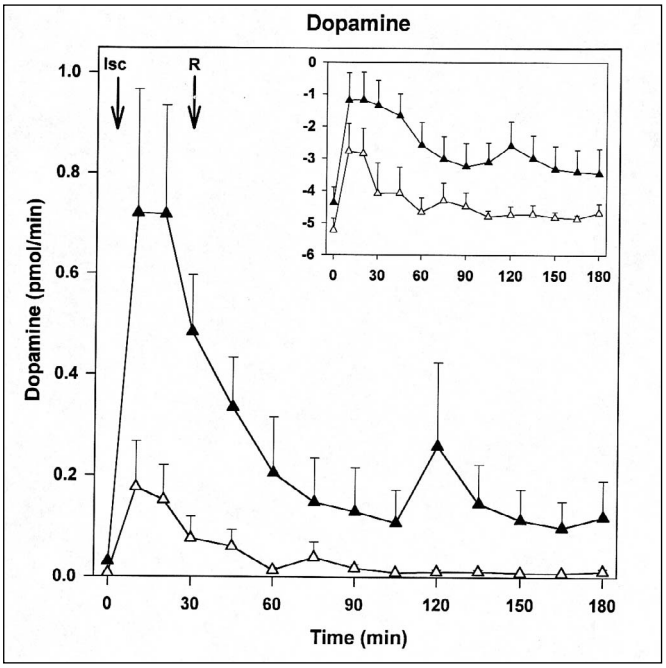


***Figure 2:*** *Striatal Dopamine Release during MCA Occlusion: The change in dopamine release in the striatum in the ipsilateral hemisphere (▲) and the contralateral hemisphere (Δ). Time* **0** *refers to the last sample collected immediately prior to ischemia induction (Isc). The third point following ischemia is the first sample collected when the contralateral CCA is released (R). Results for dopamine release in microdialysis samples are expressed as pmol/min ± SEM, n=6. Transformed data are plotted in the insert for comparative purposes.*

fixation at 24 h of recovery (n=3). Histological changes corresponding to the core and penumbra represented 34 ± 0.6% of the hemisphere at the level of bregma (data not shown).

***Metabolite release:*** Dopamine release in the lateral striatum was measured as an index of neuronal dysfunction associated with ischemia. The temporal changes in interstitial dopamine are presented in Figure 2. The change in dopamine release with time was statistically significant for both hemispheres (Time effect: p=0.0047) as was the difference between hemispheres (Hemisphere effect: p=0.0293). Initiation of ischemia produced an immediate 30-fold increase in dopamine release in the ipsilateral striatum and also a 10-fold increase in the contralateral striatum consistent with mild bilateral ischemia induced by blocking both CCAs. A similar but much smaller increase in release of dopamine and norepinephrine was observed in the ischemic cortex (data not shown).

Dihydroxyphenylacetic acid (DOPAC) release in either the cortex or lateral striatum is presented in Figure 3. Although striatal DOPAC in the ischemic hemisphere appeared to decrease during ischemia with a brief overshoot when the left CCA occlusion was reversed, there was no significant difference between hemispheres and no change over time. In contrast, cortical DOPAC release was significantly depressed during ischemia (Time X Hemisphere: p<0.0001). The decrease in DOPAC mirrored the observed increase in dopamine and norepinephrine release consistent with decreased oxidative metabolism resulting from ischemia.

***Production of 34DHB:*** The hydroxylation of 4HB to produce 34DHB was evaluated in microdialysis samples collected from either the parietal cortex or lateral striatum. Variations in 4HB availability were normalized using the 34DHB/4HB ratio. The effect of ischemia and partial reperfusion on these ratios in the cortex and striatum is presented in Figure 4. Comparison of the two regions indicates that, over all, combining data from hemispheres and times, the ratios from the striatum were significantly higher than those from the cortex (Region Effect: p=0.0008). In the striatum, the difference in the 34DHB/4HB ratio between the two hemispheres becomes smaller with time (Time X Hemisphere: p=0.0140). While the pre-ischemic ratio from the ipsilateral striatum is higher than the contralateral hemisphere, this difference did not attain significance (p=0.0517). The only individual time point that demonstrated a significant difference between the two hemispheres was at the end of the ischemic period (t=30) in the striatum (p=0.0285). This increase is slightly delayed compared to the observed increase in striatal dopamine release at the onset of ischemia.

In the cortex group, analysis of the ratios from the two hemispheres indicates a significant interaction (Time X Hemisphere: p=0.0020). However, the ratios from the two hemispheres in the cortex group at individual time points were not significantly different. Given the possibility of ischemic changes in both hemispheres due to the bilateral CCA occlusion,

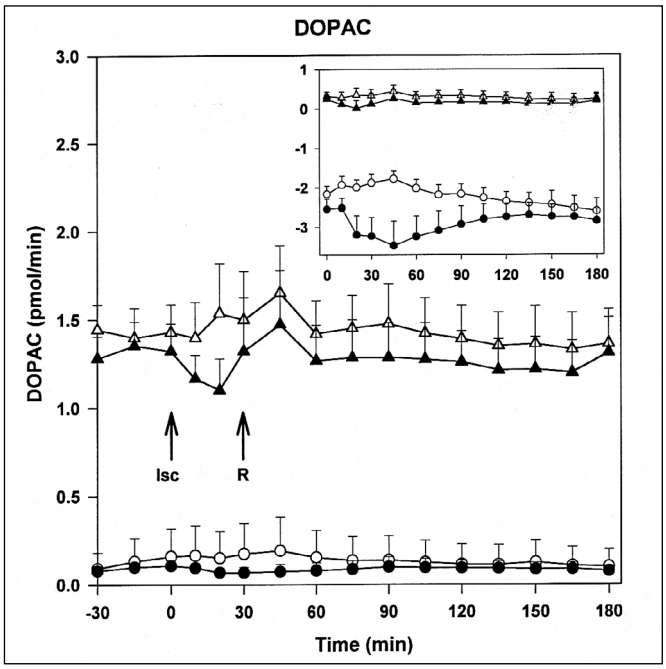


***Figure 3:*** *Striatal and Cortical DOPAC Release during MCA Occlusion: DOPAC release is presented from both the ipsilateral (●) and contralateral (O) cortex as a function of time; pre-, during and post-ischemia (lower two curves). The upper two curves present the corresponding DOPAC release from the ipsilateral (▲) and contralateral (Δ) striatum. Results for DOPAC release in microdialysis samples are expressed as pmol/min ± SEM, n=6 for both cortex and striatum. Transformed data are plotted in the insert for comparative purposes.*



https://doi.org/10.1017/S0317167100052276 Published online by Cambridge University Press

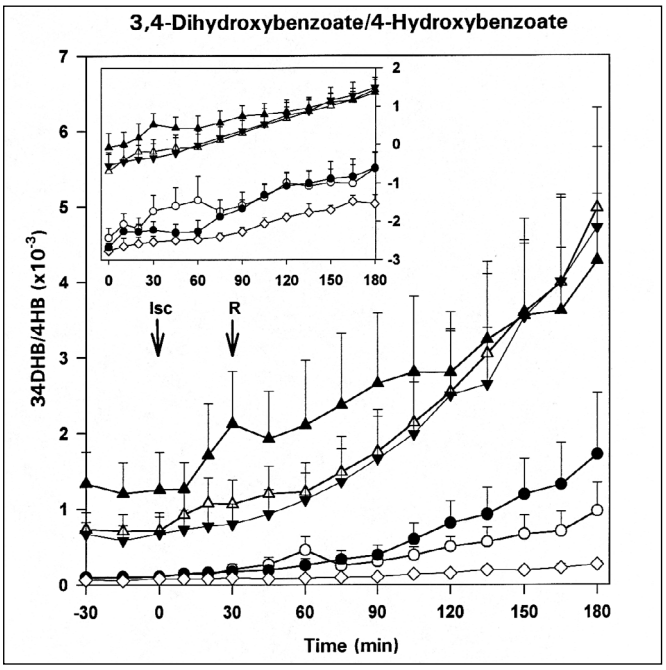


***Figure 4:*** *The Change in the 34DHB/4HB ratio in the Striatum and the Cortex during MCA Occlusion: The product to substrate ratio is presented as an index of hydroxyl radical formation. The lower three curves present the ratios from the ipsilateral (●) and contralateral (○) cortex (n=6) as a function of time; pre-, during and post-ischemia. The third curve (◊) represents the ratios from the cortex from both hemispheres (n=4) from sham-operated animals. The upper curves present the corresponding ratios from the ipsilateral (▲) and contralateral (Δ) striatum (n=6) as well as the ratios from the striatum from both hemispheres (n=4) from sham-operated animals (▼). Results are expressed as the ratio times 1000 ± SEM.*

the 34DHB/4HB ratios from the ischemic hemisphere only were compared to those from sham experiments in the cortex. When the ratios from the ischemic cortex were compared to those from sham experiments, there was a significant increase in the 34DHB/4HB ratio (Group X Time: p=0.0041), consistent with higher hydroxyl radical production in the ischemic cortex.

## Discussion

Our preliminary data using the three vessel occlusion model indicated a mild to moderate release of dopamine in the lateral striatum at the time of ischemia induction. As interstitial dopamine has been demonstrated to result in increased hydroxyl radical formation,[19,20] we wanted to evaluate hydroxyl radical production with moderate dopamine release in the striatum and compare it to production in the ischemic cortex which does not receive dopamine innervation. We have found that hydroxyl radicals are formed to a greater extent in the lateral striatum and found this to be true under all conditions (pre-ischemia, ischemia and partial-reperfusion). We have observed a similar basal production of hydroxyl radicals in an earlier study using N-methylphenylpyridinium ion to elicit dopamine release in the striatum.[32] In the present study in the ischemic cortex, hydroxyl radical production did not increase during the 30 minutes of three vessel occlusion. After the contralateral CCA release, hydroxyl radical production in both the ischemic and nonischemic cortex began to gradually increase but was more pronounced in the ischemic cortex. This corresponds to an increase in laser-Doppler readings to approximately 50% of pre-ischemic tissue perfusion. The intermediate ratios from the contralateral cortex compared to either the ischemic or sham cortex are consistent with a mild ischemic insult as suggested by the observed increase in dopamine release in the contralateral striatum during three vessel occlusion.

Salicylate trapping has been used to study hydroxyl radical formation in models of focal ischemia induced by MCA occlusion.[4-8,33] Microdialysis of the striatum,[4] the cortex[5,7,9] and of both with a horizontal probe[6,8] has shown modest increases in hydroxylation both during ischemia and following reperfusion. Comparisons have also been made between the ischemic core and penumbra in the cortex demonstrating little or no production during ischemia in the core, but increased production in the penumbral cortex (50% increase during three vessel occlusion and 250% increase at the end of 6 hour reperfusion).[9] A similar increase in the penumbral cortex had previously been reported where ischemia induced a 20% increase and reperfusion resulted in a 200% increase.[5] As determined by cresyl violet staining, the position of our probe in the frontoparietal cortex places it within the infarct core. Therefore, our observation that hydroxyl radical production did not increase during three vessel occlusion is in agreement with Solenski.[9] The increase in hydroxyl radical production during the partial reperfusion is also consistent with published data. Our study differs in that we have evaluated hydroxyl radical production in two distinct brain regions (cortex and striatum). Our finding of higher 34DHB/4HB ratios in the striatum which does not undergo infarction brings into question the role of the hydroxyl radical in cerebral ischemia and reperfusion.

The presence of large amounts of dopamine may explain why the ratio is higher in the striatum. Proximal MCA occlusion results in massive striatal dopamine release[34,35] as well as striatal infarction. It has been shown that increases in extracellular dopamine are due to neuronal dysfunction as well as inhibition of dopamine reuptake[36] associated with energy failure.[37-39] Dopamine release also occurs following $MPP^+$ administration and correlates with increased hydroxyl radical production in the striatum.[19,40] This is presumed to be caused by increased hydrogen peroxide formation from both enzymatic and nonenzymatic oxidation of dopamine. Therefore ischemia-induced release of moderate amounts of dopamine might also be expected to result in increased hydroxyl radical production. A number of studies of cerebral ischemia have demonstrated that prior dopamine depletion is neuroprotective in the striatum[41] suggesting that dopamine release contributes to neurotoxicity under ischemic conditions.[21,22] In the present study, the observed increase in the striatal 34DHB/4HB ratio after ischemia induction occurs shortly after the increase in dopamine release and is consistent with increased dopamine oxidation. In other microdialysis studies of MCA occlusion, striatal dopamine rose 3-160-fold during ischemia.[10,34,39] In one of these studies employing the three vessel occlusion model, the nonischemic contralateral striatum exhibited a 4-fold increase during ischemia[34] which we also observed. As distal MCA occlusion



https://doi.org/10.1017/S0317167100052276 Published online by Cambridge University Press

does not arrest blood flow to the striatum, we interpret the relatively small amount of dopamine released compared to that observed postmortem as an indication that the lateral striatum becomes only mildly ischemic and neither the degree of ischemia nor extent of dopamine release is neurotoxic. For comparison, in a three vessel occlusion model with proximal occlusion of the MCA that does induce striatal infarction, hydroxyl radical production in the dorsolateral striatum increased by 30% during 45 min of ischemia and by 60% following reperfusion.[4]

Although dopamine oxidation represents one mechanism, the source of hydroxyl radicals produced during ischemia/reperfusion is not clear and likely varies from region to region. In one study, transient MCA occlusion resulted in a two-fold increase in hydrogen peroxide formation (precursor to hydroxyl radical formation) in rat striatum which increased further at reperfusion.[10] However, when the ipsilateral substantia nigra underwent pre-ischemia lesioning with 6-hydroxydopamine, there was no decrease in the formation of hydrogen peroxide. This suggests that decreased dopamine availability following lesioning did not significantly alter hydrogen peroxide production and hence may not contribute to hydroxyl radical formation associated with ischemia.

If hydrogen peroxide is the precursor for hydroxyl radical formation during cerebral ischemia and reperfusion, an alternate source may be the respiratory chain as disturbed respiration is associated with superoxide production. Superoxide dismutates to form hydrogen peroxide. This source is supported by Piantadosi's work where they demonstrate that inhibitors of the respiratory chain offer neuroprotection against ischemia.[13] Clinical evidence also supports a role for superoxide in ischemic damage. Some humans with ischemic stroke have decreased serum superoxide dismutase activity compared to controls, and its activity correlated inversely with the size of infarction.[42] This is consistent with the observed increases in superoxide dismutase activity in rat brain following global ischemia[43] which can be considered to be a compensatory protective mechanism in response to increased superoxide formation. In transient focal ischemia in cats, the intravenous administration of polyethylene glycol-conjugated superoxide dismutase was protective to the caudate nucleus.[44] Recent studies using transgenic mice overexpressing extracellular superoxide dismutase have demonstrated smaller infarcts following MCA occlusion.[39] In contrast, mutations to Cu/Zn superoxide dismutase resulted in exacerbated neuronal damage following focal cerebral ischemia.[45] Similarly, knockout mice deficient in mitochondrial Mn superoxide dismutase displayed increased cerebral infarction following permanent focal cerebral ischemia.[46] These data are consistent with an increase in superoxide production resulting from ischemia and/or reperfusion leading to activation of superoxide dismutase, which is neuroprotective. Hence, in human stroke, decreased levels of circulating superoxide dismutase may reflect reduced endogenous protection, which contributes to larger infarcts. If increased production of superoxide results in its conversion to hydrogen peroxide without a concomitant increase in either catalase or glutathione peroxidase, the observed increase of tissue hydrogen peroxide during ischemia/reperfusion[10,47] provides a source for hydroxyl radical formation. This is particularly relevant in view of the increased release of iron from bound stores induced by tissue acidosis associated with ischemia where the iron ions could catalyze the decomposition of hydrogen peroxide to the hydroxyl radical via the Fenton reaction.

Other reactive species are likely involved in the ischemic injury leading to cerebral infarction and may explain the discrepancy we observe between cortical and striatal hydroxyl radical production. Among these, nitric oxide has been demonstrated to be increased during cerebral ischemia.[48,49] Recent histochemical evidence involving nitration of tyrosine residues in the ischemic zone implicates peroxynitrite,[50,51] a powerful oxidant that is formed from nitric oxide and superoxide.[52] At acidic pH, peroxynitrous acid decomposes to form an hydroxylating species that would also increase apparent hydroxyl radical production.[52] However, it is not known what proportion of peroxynitrite would decompose via this route. What is becoming increasingly clear is that, regardless of the mechanism of oxidative modification to proteins and nucleic acids, the resulting modifications can alter structure and function, thereby contributing to perturbed metabolism and cell death. The relative susceptibility of a given region to various reactive species may be quite different, resulting in greater impairment despite lower apparent production of one particular free radical.

In summary, we have examined hydroxyl radical production in both the ischemic cortex and the lateral striatum in a three vessel occlusion model of cerebral ischemia. The striatum produced higher levels of hydroxyl radicals under all conditions, which is consistent with its reported increased susceptibility to oxidative stress. These higher basal levels may set the stage for increased damage in the striatum resulting from an ischemic insult such as would be seen with proximal MCA occlusion. This might further suggest that the relatively low production of hydroxyl radicals during ischemia and even during early partial reperfusion in the ischemic cortex does not directly contribute to cell death as this region falls within the infarct volume. Alternatively, if peroxynitrite is responsible for much of the observed hydroxylation of 4HB in the ischemic cortex, we may be measuring only a small proportion of the reactive species contributing to tissue damage. Measurement of tyrosine nitration as an index of peroxynitrite formation may answer this question and this possibility is currently being investigated.

## Acknowledgments

The authors thank Drs Roger Butterworth and Alan Hazell for helpful discussions. Financial assistance is gratefully acknowledged from the Heart and Stroke Foundation of Canada (Research scholarship (JAM)) and the Medical Research Council of Canada (MT-13720), as well as the Heart and Stroke Foundation of Québec for grant support to JAM.

## References

1. Siesjö BK. Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment. J Neurosurg 1992;77:337-354.
2. Siesjö BK. Pathophysiology and treatment of focal ischemia. Part I: Pathophysiology. J Neurosurg 1992;77:169-184.
3. Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annu Rev Neurosci 1990;13:171-182.

4. Lancelot E, Callebert J, Revaud M-L, et al. Detection of hydroxyl radicals in rat striatum during transient focal cerebral ischemia: possible implication in tissue damage. Neurosci Lett 1995;197:85-88.
5. Morimoto T, Globus MYT, Busto R, et al. Simultaneous measurement of salicylate hydroxylation and glutamate release in the penumbral cortex following transient middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab 1996;16:92-99.
6. Wei J, Huang NC, Quast MJ. Hydroxyl radical formation in hyperglycemic rats during middle cerebral artery occlusion/reperfusion. Free Radic Biol Med 1997;23:986-995.
7. Yang C-S, Lin N-N, Tsai P-J, et al. In vivo evidence of hydroxyl radical formation induced by elevation of extracellular glutamate after cerebral ischemia in the cortex of anesthetized rats. Free Radic Biol Med 1996;20:245-250.
8. Wei J, Quast MJ. Effect of nitric oxide synthase inhibitor on a hyperglycemic rat model of reversible focal ischemia: detection of excitatory amino acids release and hydroxyl radical formation. Brain Res 1998;791:146-156.
9. Solenski NJ, Kwan A-L, Yanamoto H, et al. Differential hydroxylation of salicylate in core and penumbra regions during focal reversible cerebral ischemia. Stroke 1997;28:2545-2552.
10. Lei B, Adachi N, Nagaro T, et al. The effect of dopamine depletion on the $H_2O_2$ production in the rat striatum following transient middle cerebral artery occlusion. Brain Res 1997;764:299-302.
11. Landolt H, Lutz TW, Langemann H, et al. Extracellular antioxidants and amino acids in the cortex of the rat: monitoring by microdialysis of early ischemic changes. J Cereb Blood Flow Metab 1992;12:96-102.
12. Uemura Y, Miller JM, Matson WR, et al. Neurochemical analysis of focal ischemia in rats. Stroke 1991;22:1548-1553.
13. Piantadosi CA, Zhang J. Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke 1996;27:327-332.
14. Yamamoto T, Yuki S, Watanabe T, et al. Delayed neuronal death prevented by inhibition of increased hydroxyl radical formation in a transient cerebral ischemia. Brain Res 1997;762:240-242.
15. Zhang J, Piantadosi CA. Prolonged production of hydroxyl radical in rat hippocampus after brain ischemia-reperfusion is decreased by 21-aminosteroids. Neurosci Lett 1994;177:127-130.
16. Althaus JS, Andrus PK, Williams CM, et al. The use of salicylate hydroxylation to detect hydroxyl radical generation in ischemic and traumatic brain injury. Mol Chem Neuropathol 1993;20:147-162.
17. Hall ED, Andrus PK, Althaus JS, et al. Hydroxyl radical production and lipid peroxidation parallels selective post-ischemic vulnerability in gerbil brain. J Neurosci Res 1993;34:107-112.
18. Spinnewyn B, Cornet S, Auguet M, et al. Synergistic protective effects of antioxidant and nitric oxide synthase inhibitor in transient focal ischemia. J Cereb Blood Flow Metab 1999;19:139-143.
19. Chiueh CC, Krishna G, Tulsi P, et al. Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autooxidation in the caudate nucleus: effect of $MPP^+$. Free Radic Biol Med 1992;13:581-583.
20. Chiueh CC, Wu, Mohanakumar KP, et al. In vivo generation of hydroxyl radicals and MPTP-induced dopaminergic toxicity in the basal ganglia. Ann N Y Acad Sci 1994;738:25-36.
21. Globus MYT, Ginsberg MD, Dietrich WD, et al. Substantia nigra lesion protects against ischemic damage in the striatum. Neurosci Lett 1987;80:251-256.
22. Ren Y, Li X, Xu ZC. Asymmetrical protection of neostriatal neurons against transient forebrain ischemia by unilateral dopamine depletion. Exp Neurol 1997;146:250-257.
23. Ste-Marie L, Boismenu D, Vachon L, et al. Evaluation of sodium 4-hydroxybenzoate as an hydroxyl radical trap using gas chromatography-mass spectrometry and high-performance liquid chromatography with electrochemical detection. Anal Biochem 1996;67-74.
24. Bogdanov MB, Ramos LE, Xu Z, et al. Elevated "hydroxyl radical" generation in vivo in an animal model of amyotrophic lateral sclerosis. J Neurochem 1998;71:1321-1324.
25. Montgomery J, Ste-Marie L, Boismenu D, et al. Hydroxylation of aromatic compounds as indices of hydroxyl radical production: a cautionary note revisited. Free Radic Biol Med 1995;19:927-933.
26. Paxinos G, Watson C. The rat brain in stereotaxic co-ordinates. Toronto, Academic Press, 1986.
27. Chen ST, Hsu CY, Hogan EL, et al. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. Stroke 1986;17:738-743.
28. Buchan AM, Xue D, Slivka A. A new model of temporary focal ischemia in the rat. Stroke 1992;23:273-279.
29. Bederson JB, Pitts LH, Germano SM, et al. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 1986;17:1304-1308.
30. Floyd RA, Watson JJ, Wong PK. Sensitive assay of hydroxyl free radical formation utilizing high-performance liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. J Biochem Biophys Methods 1984;10:221-235.
31. Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo. Biochem J 1986;237:499-504.
32. Ste-Marie L, Vachon L, Bémeur C, et al. Local striatal infusion of $MPP^+$ does not result in increased hydroxylation after systemic administration of 4-hydroxybenzoate. Free Radic Biol Med 1999;27:997-1007.
33. Globus MYT, Ginsberg MD, Schnippering H, et al. Detection of free radical activity during ischemia by salicylate trapping and microdialysis. Stroke 1994;25:254-254.
34. Hillered L, Hallstrom A, Segersvard S, et al. Dynamics of extracellular metabolites in the striatum after middle cerebral artery occlusion in the rat monitored by intracerebral microdialysis. J Cereb Blood Flow Metab 1989;9:607-616.
35. Wood ER, Coury A, Blaha CD, et al. Extracellular dopamine in the rat striatum during ischemia and reperfusion as measured by in vivo electrochemistry and in vivo microdialysis. Brain Res 1992;591:151-159.
36. Akiyama Y, Ito A, Koshimura K, et al. Effects of transient forebrain ischemia and reperfusion on function of dopaminergic neurons and dopamine reuptake in vivo in rat striatum. Brain Res 1991;561:120-127.
37. Chang CJ, Ishii H, Yamamoto H, et al. Effects of cerebral ischemia on regional dopamine release and $D_1$ and $D_2$ receptors. J Neurochem 1993;60:1483-1490.
38. Damsma G, Boisvert DP, Mudrick LA, et al. Effects of transient forebrain ischemia and pargyline on extracellular concentrations of dopamine, serotonin, and their metabolites in the rat striatum as determined by in vivo microdialysis. J Neurochem 1990;54:801-808.
39. Obrenovitch TP, Richards DA. Extracellular neurotransmitter changes in cerebral ischemia. Cerebrovas Brain Metab Rev 1995; 7:1-54.
40. Obata T, Chiueh CC. In vivo trapping of hydroxyl free radicals in the striatum utilizing intracranial microdialysis perfusion of salicylate : effects of MPTP, $MPDP^+$, and $MPP^+$. J Neural Transm 1992;89:139-145.
41. Clemens JA, Phebus LA. Dopamine depletion protects striatal neurons from ischemia-induced cell death. Life Sci 1988;42:707-713.
42. Spranger M, Krempien S, Schwab S, et al. Superoxide dismutase activity in serum of patients with acute cerebral ischemic injury. Correlation with clinical course and infarct size. Stroke 1997;28:2425-2428.
43. Sutherland GR, Bose R, Louw DF, et al. Global elevation of brain superoxide dismutase activity following forebrain ischemia in rat. Neurosci Lett 1991;128:169-172.
44. Matsumiya N, Koehler RC, Kirsch JR, et al. Conjugated superoxide dismutase reduces extent of caudate injury after transient focal ischemia in cats. Stroke 1991;22:1193-1200.
45. Kondo T, Reaume AG, Huang T-T, et al. Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia. J Neurosci 1997;17:4180-4189.

46. Murakami K, Kondo T, Kawase M, et al. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. J Neurosci 1998;18:205-213.
47. Lei B, Adachi N, Arai T. The effect of hypothermia on $H_2O_2$ production during ischemia and reperfusion: a microdialysis study in the gerbil hippocampus. Neurosci Lett 1997;222:91-94.
48. Malinski T, Zhang ZG, et al. Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 1993;13:355-358.
49. Kader A, Frazzini VI, Solomon RA, et al. Nitric oxide production during focal cerebral ischemia in rats. Stroke 1993;24:1709-1716.
50. Keller JN, Kindy MS, Holtsberg FW, et al. Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J Neurosci 1998;18:687-697.
51. Coeroli L, Arnaud RS, Plotkine D, et al. Nitric oxide production and perivascular tyrosine nitration following focal ischemia in neonatal rat. J Neurochem 1998;70:2516-2525.
52. Koppenol WH. The basic chemistry of nitrogen monoxide and peroxynitrite. Free Radic Biol Med 1998;25:385-391.



https://doi.org/10.1017/S0317167100052276 Published online by Cambridge University Press