Binding of the Hypoxia Tracer \[^{3}H\]Misonidazole in Cerebral Ischemia

John M. Hoffman, Janet S. Rasey, Alexander M. Spence, Dennis W. Shaw, and Kenneth A. Krohn

Radiolabelled misonidazole, a radiosensitizing drug that binds to viable hypoxic tumor cells, may be useful in identifying hypoxic cells in cerebrovascular disease. Its potential was investigated in the gerbil stroke model. Biodistribution of \[^{3}H\]misonidazole was measured in normal gerbils and animals that had been subjected to right common carotid artery ligation to produce cerebral ischemia. The uptake of \[^{3}H\]misonidazole in the right cerebral hemisphere and right/left hemispheral uptake ratios correlated positively with the severity of the stroke when measured 6–9 hours after carotid ligation. Histologic studies in symptomatic ligated animals showed ipsilateral widespread but patchy acute ischemic changes as well as areas that showed no morphological changes. Microscopic autoradiography in these animals showed diffuse heavy labelling only in the ipsilateral hemisphere. This was over areas that had histological damage as well as in adjacent areas that appeared intact. Studies comparing blood flow measured with \[^{14}C\]iodoantipyrine and \[^{3}H\]misonidazole retention in gerbils with carotid artery ligations indicated that flow is not a major determinant of retention of this hypoxia tracer. We conclude that a misonidazole congener labelled with a gamma- or positron-emitting isotope may be useful in nuclear imaging of the degree and regional distribution of hypoxic tissue in cerebrovascular disease. (Stroke 1987;18:168–176)

Radiation biologists have investigated the potential for imaging hypoxic tumor tissue with nitroimidazole derivatives, a class of drugs that sensitize hypoxic tumor cells to ionizing radiation. Misonidazole, the most thoroughly studied nitroimidazole, binds covalently to viable hypoxic tumor cells in rodent solid tumors and in tissue culture monolayers or spheroids. It does not bind in necrotic, irreversibly damaged tumor cells and may therefore serve as a marker of sublethal hypoxic cellular injury. To date, there have been no investigations of misonidazole uptake in hypoxia associated with cerebral ischemia. We report here such a study in the gerbil stroke model, which was initially described by Levine and Payan. Unilateral carotid occlusion produces homolateral ischemia and/or infarction in approximately 30–50% of adult male animals because of anatomic variations in the circle of Willis.

Our studies of the biodistribution of radiolabelled misonidazole in gerbils with or without carotid ligation showed preferential uptake of the compound in the ischemic hemisphere of those animals with clinical evidence of cerebral ischemia. These results were confirmed using microscopic autoradiographic techniques. Based on these encouraging initial findings, further work is underway to produce a radiolabelled misonidazole congener to function as a positron emission tomography imaging agent of hypoxia in cerebrovascular disease.

Materials and Methods

Radiolabelled Misonidazole

\[^{3}H\]Misonidazole has been synthesized in our laboratory according to the method of Born and Smith by reduction of 8 mg of the precursor ketone 1-(3-methoxy-2-oxopropyl)-2-nitroimidazole with sodium \[^{3}H\]borohydride (Amersham, 8.4 mCi/mmol). This synthesis produces a tritium atom on the side chain at the second carbon from the imidazole ring. The radiochemical purity of the product was greater than 98%, determined by high performance liquid chromatography (HPLC). The first lot crystallized from the reaction mixture had a specific activity of 77 mCi/mmol (383 \(\mu\)Ci/mg); a second lot had a specific activity of 6 mCi/mmol (29.7 \(\mu\)Ci/mg).

Animals and Surgical Procedures

Adult male gerbils (Meriones unguiculatus) weighing 66–122 g were obtained from Tumblebrook Farms (West Brookfield, Mass.) and housed in controlled animal facilities for a minimum of 2 weeks prior to any studies. They were given food and water ad libitum.

To produce cerebral ischemia a right common carotid ligation was performed after anesthesia with intraperitoneal (i.p.) ketamine (40 mg/kg) or inhalant penthrane. With inhalant penthrane, induction and recovery were more rapid and there was less intraoperative mortality. Hence, this became our standard agent.
A midline anterior cervical incision was made. The right common carotid artery was isolated and ligated with 6-0 silk suture distally and proximally. The vessel was then transected to assure no flow. Thereafter, the incision was closed, the animals allowed to awaken, and the stroke index described by Ohno et al (Table 1) was calculated at 15, 30, 45, 60, 90, 120, 180, and 240 minutes after carotid ligation. When animals were monitored longer than 4 hours, stroke indices were determined at 1- to 4-hour intervals. Prior to sacrifice of any animal, a final stroke index was calculated.

### Table 1. Modified Stroke Index

<table>
<thead>
<tr>
<th>Signs</th>
<th>Stroke index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair roughed up to tremor</td>
<td>1</td>
</tr>
<tr>
<td>Obtunded or paucity of movement</td>
<td>1</td>
</tr>
<tr>
<td>Hypoesthesia of ear</td>
<td>1</td>
</tr>
<tr>
<td>Head cocked</td>
<td>3</td>
</tr>
<tr>
<td>Eye fixed open</td>
<td>3</td>
</tr>
<tr>
<td>Ptosis</td>
<td>1</td>
</tr>
<tr>
<td>Splayed-out hind limb</td>
<td>3</td>
</tr>
<tr>
<td>Circling</td>
<td>3</td>
</tr>
<tr>
<td>Seizures or abrupt explosive movement</td>
<td>3</td>
</tr>
<tr>
<td>Extreme weakness</td>
<td>6</td>
</tr>
</tbody>
</table>

**TOTAL**

25

Modified stroke index from Reference 12.

Histology

Eight animals were anesthetized with inhalant penthrane and then subjected to right common carotid ligation to correlate histologic changes and the stroke index after varying periods of ischemia. These animals did not receive [3H]misonidazole. After the 240-minute calculation, the index was obtained at arbitrary times but always just prior to sacrifice or death. The minimum sacrifice time post-ligation was 18.5 hours and the maximum, 46 hours. At death or sacrifice, the brain was removed and fixed in 10% neutral buffered formalin, then embedded in paraffin. Coronal sections were prepared for histology and stained with hematoxylin and eosin or Luxol fast blue with periodic acid-Schiff (PAS).

**Autoradiography**

Brains from another series of carotid-ligated animals were prepared for [3H]misonidazole microscopic autoradiography to measure the microscopic distribution of the compound in ischemic and nonischemic brain regions. Carotid ligation was performed, and the stroke index was calculated at the usual times. Animals with a stroke index of > 10 were placed in the multiple injection protocol with [3H]misonidazole (specific activity of 29.7 μCi/mg). Two animals were sacrificed 2 hours after the final injection of drug, which was between 5 and 6 hours after ligation. The brains were cut into 6 coronal sections, each 3 mm thick. To compare the left and right hemispheres, alternate sections were cut sagittally in the midline and processed for liquid scintillation counting as previously described to allow calculation of %ID/g. The remaining control sections were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm. Autoradiographs were prepared with Kodak NTB emulsion (Kodak, Rochester, N.Y.), exposed for 6 months, and developed.

To compare [3H]misonidazole uptake and blood flow in ischemic gerbil brains, 8 male gerbils were subjected to right carotid artery ligation as described above. Two hours after ligation, a single i.p. injection of [3H]misonidazole (50 μmol/kg) was given. At 4 hours post-ligation and 10 seconds before sacrifice, a bolus injection of [14C]iodoantipyrine (Amersham, specific activity 59 mCi/mmol) was administered through a femoral vein catheter as a blood flow tracer. The catheter had been implanted 24 hours prior to carotid ligation. After sacrifice, multiple samples of brain from both the right and left hemispheres were processed differently from those of control animals by subsectioning to determine the uptake in different regions of the ischemic hemisphere. On sacrifice, the whole brain was removed, placed on powdered dry ice for 30 seconds, and then sliced coronally at approximately 2-mm intervals, yielding a total of 7 coronal slices. Each slice was then cut in half in the midsagittal plane, and the right and left halves were placed in preweighed, labelled scintillation vials. Using standard techniques, the %ID/g was calculated for all brain slices.
FIGURE 1. Biodistribution of [³H]misonidazole in blood and non-CNS tissues of control gerbils and animals subjected to right common carotid artery ligation and in brains of control gerbils. Uptake in brains of ligated animals is shown separately for each animal in Figure 2. Animals received 3 i.p. injections of [³H]misonidazole (50 μmol/kg) within 2 hours and were sacrificed 2 hours after the last injection. Each bar is the mean value of tissue uptake for 5 animals, and the lines are SEM.

Results

The organ biodistribution data for both normal (unligated) and ligated animals are presented in Figure 1. In control animals the %ID/g for whole brain (0.137 ± 0.017) and blood (0.175 ± 0.025) were similar. The brain/blood ratio was 0.80 ± 0.04. Liver, gut, and kidney showed two- to threefold greater uptake than blood. For non-CNS tissues and blood there were no significant differences between ligated and control animals, and therefore these data were compared directly. The brain data for ligated vs. control animals were not directly compared due to the difference in tissue preparation. From control animals a single sample of the mid-right hemisphere was taken (Figure 1), while brains from ligated animals were cut into serial coronal sections (Figure 2) as described in "Materials and Methods." Thus, no brain uptake data for ligated animals are shown in Figure 1.

The increased uptake of [³H]misonidazole in ischemic hemispheres is shown in Figure 2, which contains brain data on 5 animals with stroke indices ranging from 0 to 13. There was fairly uniform uptake and no oxidized to separate carbon-14 and tritium; these samples were counted in a liquid scintillation spectrometer. Average [¹⁴C]iodoantipyrine uptake into the multiple samples of the unaffected left hemisphere was normalized to 1.0, and the relative blood flow (uptake of carbon-14) into individual samples from the right or left brain was expressed as a percent of this value. [³H]misonidazole uptake was calculated as %ID/g for each sample.
right–left differences in any coronal sections in the animal with no evidence of ischemia (stroke index = 0) and minimal right–left differences in the animal with a stroke index of 4. There was no significant difference in brain misonidazole uptake in all 7 coronal brain slices from ligated gerbils with low stroke index (Figure 2) when compared with control animals (Figure 1). The 2 animals which were markedly symptomatic (stroke index = 13) at the time of sacrifice showed a two-to-threefold higher uptake in the mid-parietal region of the right hemisphere when compared with the left. The increased uptake in the anterior regions of the brain in the other symptomatic animal (stroke index = 10) was positively correlated with stroke index. The left brain of the most symptomatic animals also showed a one- to twofold increase in uptake when compared with similar coronal sections in animals with very low stroke index.

Blood clearance of [3H]misonidazole was calculated for control and ligated animals. Both groups received multiple [3H]misonidazole injections as described above for the animals in the biodistribution studies (Figure 3). The clearance curves had the same slope in both groups of animals. Linear regression analysis yielded two separate best-of-fit curves, but there was no significant difference in %ID/g of blood samples between control and ligated animals at any sample time.

Several animals were subjected to ligation and sacrificed at 18–46 hours in an attempt to compare histologic changes with time and stroke index. There were no findings in right hemispheres of gerbils with stroke indices of 10 or less and no findings in any left hemisphere. Animals with scores of 11–17 showed extensive patchy acute infarction in the right hemisphere from the anterior limit of the corpus striatum to the level of the upper mesencephalon, which included the major structures: cerebral cortex, corona radiata, internal capsule, corpus striatum, globus pallidus, thalamus, and upper mesencephalic tegmentum. In the frankly damaged tissue areas, the neuropil demonstrated pallor of staining and widespread spongiform changes. Neurons showed ischemic cell change with shrunken hyperchromatic nuclei and cell bodies, or early karyorrhexis (Figure 4). All of the above structures also contained areas that lacked histological changes, i.e., either had not undergone irreversible necrosis or had done so but failed to evolve the morphological changes in the short time span of our experiments.

In the 2 animals in which the brain was prepared for autoradiography, the final stroke indices were 17 and 14 and were never less than 10 at any observation time. These animals were sacrificed 2 hours after the third injection and approximately 6 hours after ligation. There was intense labelling in the ischemic right hemisphere and only sparse labelling in the left. The labelling was relatively evenly spread over all right hemispheric areas, which included regions that did not show the histological changes of acute ischemia as well as those that did (Figure 5). There did not appear to be any tendency for labelling to concentrate over nuclei, cytoplasm, vessels, neuropil, or any particular cell type.

Alternate coronal brain slices from these 2 animals were also obtained and processed for liquid scintillation counting. This confirmed that there was greater uptake of [3H]misonidazole in the right hemisphere compared with the left (Figure 6). The accumulation of [3H]misonidazole in the anterior regions of the brain in these 2 symptomatic animals and in 4 of the 5 animals shown in Figure 2 verifies that there is a trend for increased uptake with increasing stroke index (Figure 7).

Figure 8 compares the [3H]misonidazole uptake and relative blood flow for different areas of the brains of 3 representative gerbils subjected to right carotid artery ligation. The presacrifice stroke index for each animal is listed in the figure. On the left side of the brain, there was variation in blood flow from 65 to 130% of the mean value for that hemisphere but essentially no variation in [3H]misonidazole uptake. In symptomatic ligated animals, relative flow on the right side was reduced. There was no consistent indication of variation in misonidazole retention as a function of varying flow
FIGURE 4. Section of right cerebral gray matter from a gerbil with stroke index of 10 at the time of sacrifice, 30 hours after ligation. This animal did not receive [3H]misonidazole. The section shows pallor and spongiform changes in the right half and acute ischemic cell changes in the left half (HE stain; ×100).

Discussion

Our studies were designed to test whether there was increased binding of [3H]misonidazole in ischemic brain that could be detected by autoradiography or liquid scintillation counting (LSC) and eventually by nuclear imaging and positron emission tomography. The LSC data show a two- to eightfold increase in tracer concentration in ischemic brain compared to contralateral normal brain. The quantity of labelling correlates with the stroke index (Figures 2, 6, and 7). The autoradiographs confirm this finding and show that the uptake is diffuse and evenly distributed throughout the hemisphere ipsilateral to the ligation (Figure 5). In gerbils with a low stroke index there was no greater uptake in the right hemisphere compared with the left.

The rationale for this work stemmed from experience and evidence gathered on misonidazole binding under conditions of hypoxia in vitro using cells in culture and in vivo using experimental rodent tumors. In monolayer cultures, misonidazole binds to anoxic but not fully oxygenated cells, and the binding rate increases as O2 concentration in the medium decreases from 180 to 2 μM (122 to 1.4 mm Hg). Miller et al concluded that misonidazole is covalently bound to cellular macromolecules in hypoxic EMT-6 mouse sarcoma cells in vitro, and the temperature dependency of binding strongly suggests that this is an enzymatic process. In vitro, 0.7-1 mm diam. V-79 and EMT-6 mouse sarcoma cell spheroids in fully oxygenated medium (Po2 = 145 mm Hg) bind about 50-fold more misonidazole in the viable rim of cells around the necrotic center than in the well-oxygenated periphery or the dead necrotic center. Electrode measurements have shown that Po2 in spheroids of this size decreased from 100-130 mm Hg at the spheroid surface to 0-3 mm Hg at or near the center of V-79 spheroids and 5-25 mm Hg in EMT-6 spheroids. Several mouse tumors in vivo behave similarly, with most radiolabel bound in cells directly adjacent to necrosis, which occurs 150-200 μm from blood vessels and is presumed due to insufficient O2 for cell growth (J.S. Rasey, unpublished observations). There is virtual absence of label in necrotic areas of tumors as well as in necrotic centers of larger spheroids, even when these areas are directly adjacent to viable-appearing cells with heavy labelling. This strongly argues that nonviable hy-
FIGURE 5. Top. Microscopic autoradiograph of [3H]misonidazole uptake from the right thalamus of a gerbil with stroke index of 14 at death, 6 hours after right carotid ligation. Note the density of the silver granules and the shrunken dark-staining ischemic cellular nuclei. Whole field shows ischemic change (HE stain; ×400). Center. Autoradiograph from right hemisphere of same animal as in 5 top. This shows heavy labelling over an area with minimal-to-no histologic change (HE stain; ×400). Bottom. Section similar to 5 top from the contralateral uninfarcted thalamus of the same gerbil to show the very sparse tritium-developed granules and large pale normal neuronal nuclei (HE stain; ×400).
poxic tissue cannot bind the labelled drug because the required enzymatic reductions cannot occur in dead cells.

Ours is the first investigation that applies this knowledge to a stroke model with the eventual goal to regionally image in human tissue sublethal ischemia and hypoxia with a positive radionuclide marker. That our present results bring us closer to this goal is argued as follows.

Prior work by Ohno et al has established convincingly that there is a 5- to 12-fold reduction in ipsilateral cerebral blood flow (rCBF) on the ligated side in gerbils with stroke indices of > 10 and a lesser and variable reduction in animals with stroke indices ranging from 3 to 9.12 Kelly and Halsey used the platinized platinum electrode to measure simultaneous rCBF and oxygen availability (O₂a) at a single locus in the gerbil brain.17 They generally found immediate simultaneous reduction in both parameters at several anatomic sites after temporary carotid ligation, although the correlation was better in some animals than others. These results suggest a correlation between increasing stroke index, reduced blood flow, and reduced O₂a. Our data show a positive correlation between misonidazole uptake and stroke index (Figure 7) and also indicate that misonidazole can enter and be retained in ischemic brain tissue despite reduced blood flow (Figure 8).

The measurements of relative blood flow and mis-
Misonidazole uptake (Figure 8) indicate that the entry of this lipophilic radiolabelled drug into ischemic brain and its retention are not principally determined by flow. Data obtained on blood flow, measured with rubidium-103 microspheres vs. $[{}^3{}H]$misonidazole retention in a dog myocardial infarct model have shown a threefold variation in flow but constant misonidazole uptake in the noninfarcted region and an inverse relation between flow and misonidazole binding in the infarcted tissue (J. Caldwell and G. Martin, Seattle VAH, personal communication). While these studies do not rule out a role for drastically reduced blood flow in limiting the uptake of misonidazole into ischemic regions, they suggest that the delivery and retention of such a lipophilic drug will be principally determined by other parameters. O$_2$ level is likely to be the most important factor based on the available evidence from other systems.

This interpretation is further supported by evidence reported by Horowitz et al. They studied $[^{14}C]$misonidazole distribution in the RT-9 rat brain tumor model and correlated this with rCBF by means of double-label quantitative autoradiography (QAR). In normal gray matter they found uniform distribution of low levels of misonidazole, with approximately 10–15% lower values in normal white matter at 0.5, 2, and 4 hours after injection. In tumor implants there was no consistent correlation between rCBF and regional carbon-14 concentration. In fact, tumor-to-normal brain ratios of carbon-14 increased over the experimental period despite the finding that tumor blood flow was lower than normal brain blood flow. From these findings it was concluded that reduced blood flow in tumors could not explain the distribution of the compound.

It is equally unlikely that misonidazole is retained in ischemic brain solely because reduced flow retards its washout. Flow in the brains of gerbils with severe infarcts still is about 8–21% of control levels; it does not fall to zero. Also, the accumulated labelled misonidazole in ischemic gerbil brain remains bound through all procedures required to prepare autoradiographs. This demonstrates conclusively that the compound is tightly and probably covalently bound.

Because misonidazole is lipophilic, its entry into the brain is probably not limited by the blood–brain barrier, nor does breakdown of the barrier explain entry of drug into the brain of gerbils with infarcts. One reason for this is that the barrier in gerbils remains largely intact for up to 17 hours after ligation, as determined by the ability of the brain to exclude trypan blue.

In addition, Brown and Workman studied the effect of lipophilicity on distribution of misonidazole and several other nitroimidazoles in BALB/c mice. The brain/plasma ratio was essentially 1.0 for drugs with an octanol/water partition coefficient of $>0.25$. Misonidazole has a partition coefficient of 0.43. Consistent with our results in both control and ligated gerbils, these investigators reported brain/plasma ratios of misonidazole of 1.0 at 20, 45, and 75 minutes after a single i.p. injection. At 2 hours after the last of 3 injections we found blood values that were essentially the same in ligated as in control animals. The brain/blood ratios range from 0.71 to 0.91 in control animals and in those ligated gerbils with a low stroke index. Our data show a rapid clearance of misonidazole from the blood of both ligated and nonligated gerbils after the multiple injection protocol with essentially no difference in clearance rates between the two groups. The blood clearance can be described by a monoexponential function over the 2-hour experimental period. Thus, misonidazole demonstrates the expected pattern of washout from normal (nonischemic) brain of a lipophilic, freely diffusible compound.

The current data do not clarify whether misonidazole binds above or below the level of hypoxia, at which normal brain tissue cell viability is destroyed. A.J. Franko et al (unpublished observations) determined that for several tumor types in short-term organ culture, $[^{14}C]$misonidazole binding was inhibited approximately 50% by O$_2$ levels of 2,000–9,000 ppm (1.5–6.8 mm Hg) relative to binding levels occurring under anoxic conditions. With our present data it also is not possible to distinguish whether misonidazole accumulated 1) in cells that were hypoxic but not yet irreversibly damaged at a time preceding necrosis, or 2) in cells that were already irreversibly damaged at the time the misonidazole reached them. The regions in the ipsilateral hemisphere showing heavy label over histologically preserved cells and neuropil (Figure 5 Center) suggest that misonidazole does bind in sublethally hypoxic cells. Although we cannot rule out that these regions represent irreversibly injured cells that had not evolved the histologic changes of ischemia, the absence of binding in necrotic cells of tumors or the in vitro tumor model, multicell spheroids, argues against frankly dead cells being able to bind the drug. In addition, labelled histologically damaged cells seen 6–9 hours after carotid ligation may have been viable when labelled drug was first available but died subsequently. Misonidazole binds relatively evenly throughout the underperfused tissue, not just at margins where perfusion is preserved. This distribution of label in autoradiographs suggests that there may be a distinct advantage to using such a compound to produce a positive image of the regional distribution of sublethally damaged hypoxic tissue rather than a negative image of the preserved tissue that surrounds a hypoxic area. However, more investigations are required to determine whether misonidazole is detecting reversible hypoxia in the brain.

The coronal brain sections (Figures 2 and 6) consistently showed a decreasing rostral–to–caudal binding of label. Anatomical features of gerbil cerebral vessels may explain this greater uptake in the forward one-third of the right hemisphere and also why markedly symptomatic animals (stroke index of $>13$) had increased uptake in the anterior regions of the left hemisphere. The gerbil has an incomplete circle of Willis and, depending on collateral flow, may develop ischemia after carotid ligation. The propensity to develop ischemia depends on the variability of small connect-
ing vessels between the vertebro–basilar and carotid circulations. The gerbil also has a unique anterior circulation since the 2 anterior cerebral arteries fuse in the interhemispheric fissure to form a single pericallosal artery. 6,4 Ischemia may well have been present in the anterior brain regions on the nonligated side secondary to the loss of the contributing anterior cerebral flow from the opposite internal carotid circulation. The cerebellum, which is supplied by the posterior circulation, was included in the 2 most posterior sections where there was essentially no difference between left and right.

Our biodistribution data in non-CNS organs were consistent with findings from other laboratories. 21,22 Liver and kidney showed higher uptake than most other normal tissues. The reason for high retention in liver may relate to misonidazole being metabolized there by nitroreductase enzymes or by conjugation prior to excretion. Alternatively, liver may contain hypoxic foci. 23 The gerbil has a unique concentrating kidney that may be responsible for increased uptake in addition to major excretion through the urinary tract. In preparation of the bowel for analysis the contents were not removed, and anaerobic bacteria may be responsible for the increased uptake seen in this organ, especially after i.p. injection.

The lipophilicity and hypoxia-mediated binding properties of misonidazole make it a potentially useful agent for imaging cerebral ischemia. Based on these unique properties and our preliminary results, we are actively investigating a positron-emitting congener that can be applied to the study of ischemia and hypoxia in man.

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