Characterization of Fluoromisonidazole Binding in Stroke

Neil J. Spratt, BMSci, FRACP; Uwe Ackerman, PhD; Henri J. Tochon-Danguy, PhD; Geoffrey A. Donnan, MD, FRACP; David W. Howells, PhD

Background and Purpose—[18F]fluoromisonidazole (FMISO) positron emission tomography has been used to image hypoxia early after human stroke. To further study the role of hypoxia in stroke and the binding characteristics of FMISO, we aimed to develop [3H]FMISO autoradiography in an animal stroke model. We hypothesized that [3H]FMISO binding is prolonged, allowing correlation with 24-hour histology, and that there is no FMISO binding after effective reperfusion.

Methods—Temporary middle cerebral artery (MCA) occlusion was performed in rats, followed by [3H]FMISO administration. Tissue preparation for autoradiography and histology (from the same sections) was performed 2.5 hours after MCA occlusion (MCAo; replicating [18F]FMISO studies). Then, otherwise identical cohorts with tissue preparation at 2.5 or 24 hours were prepared. For reperfusion studies, animals had 1-hour MCAo, with [3H]FMISO administered 1 hour after reperfusion.

Results—[3H]FMISO autoradiography provided a high-resolution image of hypoxia throughout the ischemic territory. Delaying animal death from 2.5 to 24 hours allowed histological changes of stroke to develop, without significantly altering either relative intensity (1.88 ± 0.06 and 2.02 ± 0.11, respectively) or volume (25 ± 6 mm³ and 28 ± 5 mm³, respectively) of hypoxic binding. [3H]FMISO binding did not occur after effective reperfusion, despite histological injury from the preceding MCAo.

Conclusions—[3H]FMISO autoradiography of hypoxia in experimental stroke offers several advantages. Bound FMISO is retained in tissues long term, enabling direct correlation with 24-hour histology. It is not bound after effective reperfusion. Therefore, positive [18F]FMISO positron emission tomography studies in stroke patients are indicative of ongoing tissue hypoxia, not merely recent tissue injury. (Stroke. 2006;37:1862-1867.)

Key Words: animal models ■ cerebral infarction ■ pathology ■ stroke, acute

Fluoromisonidazole (FMISO) is a nitroimidazole compound that has been used to image hypoxia in tumors, ischemic myocardium, and stroke.1-6 [18F]FMISO positron emission tomography (PET) of stroke patients has been investigated as a possible penumbral imaging method, which, because of the direct imaging of cellular hypoxia, may overcome some of the difficulties with existing indirect perfusion-based imaging methods. This may be particularly relevant in tissues with lower baseline perfusion, such as cerebral white matter.7 Binding has been shown to occur in a peri-infarct distribution over a time course that would be consistent with penumbral binding, and some of the bound tissue has been shown to progress to infarction, whereas some is salvaged.3,4 Importantly, the proportion of bound tissue progressing to salvage correlates well with improvements in clinical severity as measured by the National Institutes of Health Stroke Scale, Barthel index, or modified Rankin score.3,5 There are a number of unresolved questions regarding the use of FMISO that cannot be easily answered in human subjects, including confirmation that reperfused tissue does not bind FMISO.

We previously reported the feasibility of [18F]FMISO autoradiography in a rat stroke model; however, synthesis of a tritiated form of the probe ([3H]FMISO) has facilitated high-resolution animal autoradiographic studies. Histological change after stroke takes ≥24 hours to evolve, and therefore, most techniques to investigate ischemia and hypoxia in vivo have had to rely on separate animal cohorts for tissue outcome.8 Because of the very prolonged half-life of tritium and reported prolonged binding of FMISO irrespective of subsequent tissue oxygenation, we hypothesized that tissue preparation and autoradiography may be able to be delayed for 24 hours without significant loss of bound signal, thus allowing study of hypoxia and histology in the same tissue. Our aims were to establish the feasibility of [3H]FMISO for imaging hypoxia in a rat stroke model and to determine whether tissue processing 24 hours after [3H]FMISO administration was possible without significant loss of autoradiographic signal. Finally, we sought to determine whether FMISO binding occurs after reperfusion.
Methods

Animal Preparation
All animal experimentation was performed with the approval of the Austin health animal ethics committee and in accordance with guidelines of the National Health and Medical Research Council. Twenty-seven Sprague-Dawley rats weighing 280 to 320 g were used. Anesthesia was induced with 5% isoflurane in an induction box and maintained with 2% isoflurane administered in a 50:50 air/oxygen mix. A total of 120 μg of atropine was administered intraperitoneally immediately after induction. Blood oxygen saturation and heart rate were monitored throughout the procedure (pulse-oximeter; Nellcor), and temperature was maintained at 37°C using a locally manufactured rectal temperature-regulated heating pad and heating lamp. Blood pressure and serum glucose were not monitored.

Stroke Induction
Blood flow over the ischemic hemisphere was monitored via continuous laser Doppler flowmetry (Moor Instruments). A Moorlab D5probe was mounted over the thinned skull 1 mm posterior and 2 mm lateral to Bregma using a laboratory-manufactured rubber probe holder fastened to the skull with Loctite 406 instant adhesive. Middle cerebral artery (MCA) thread occlusion for the initial comparison with [18F]FMISO was performed using poly-l-lysine–coated 4/0 nylon monofilament-occluding suture as described previously.6 For subsequent experiments, silicone-tipped 3/0 monofilament-occluding sutures were used because we have shown these to produce more consistent infarction with lower mortality.9 The surgical technique was unaltered and is described below.

After ligation of branch arteries including the pterygopalatine artery and creation of an external carotid artery stump, the thread was passed via the right external carotid and up the internal carotid artery 18 to 20 mm, depending on animal size, until resistance was felt and a sudden decrement in flow was seen by skull-mounted laser Doppler flowmetry. Experimental animals were subjected to different intervals of MCA occlusion (MCAo), and then the MCA-occluding suture was retracted into the external carotid stump under anesthesia to reopen the MCA, with confirmation of reperfusion made by laser Doppler flow monitoring. For subsequent analysis, laser Doppler flow signals were averaged over 5-minute epochs immediately before and after MCAo and reperfusion and were assessed as a percentage of baseline.

Tissue Processing and Histology
After the required survival interval, animals were anesthetized with isoflurane in an induction box, and then cardiac perfusion fixation was performed with 4% paraformaldehyde after washout with 0.9% saline. Brains were postfixed in paraformaldehyde for 48 hours, then placed in 30% sucrose for 2 days before snap-freezing in liquid isopentane on dry ice. Brains were stored at −80°C until sectioning at 40 μm on a cryostat (Microm). Sections were thaw-mounted onto gelatin-coated microscope slides, with every sixth section collected. These slides were stained with hematoxylin and eosin (H&E) before autoradiography and were subsequently overslipped for histological analysis. Slides were scanned on an Epson expression 1600 flatbed scanner (Seiko Epson), and 1000 dots/inch red, green, blue color images were analyzed using the MCID M2 software package (Imaging Research). Area of infarction was assessed by drawing an area of increased binding and that the threshold did not detect binding within the nonischemic hemisphere.

[3H]FMISO Autoradiography
In brief, 1-(3-fluoro-2-oxopropyl)-2-nitro-1H-imidazole was synthesized as described by Grunbaum et al.10 To a solution of 4.5 mg of 1-(3-fluoro-2-oxopropyl)-2-nitro-1H-imidazole (0.024 mmol/L) in absolute ethanol (200 μL) was added tritiated NaBH3 (100 μCi, 16 Ci/mmol/L, 0.00625 mmol/L). The reaction mixture was stirred for 2 hours at room temperature and subsequently hydrolyzed with 100 μL of 1 mol/L HCl. After addition of 2 mL of H2O, [3H]FMISO was trapped on a Waters C-18 SepPak column, the SepPak washed with 5 mL of H2O, and the product eluted with 1 mL ethanol. The end product was administered by tail-vein injection either 30 minutes or 2 hours after MCAo as specified. Autoradiography was performed by exposing sections to BAS-TR2025 phosphor-imaging plates for 14 days. Plates were read in a BAS-5000 plate reader (Fuji) and analyzed using the MCID software package. For alignment with the corresponding histological sections, a fiducial marker system was developed using a 3% gelatin solution with food dye and 1:1000 [1H]FMISO. Small dots were placed around each section using a fine pipette tip, and the slides air dried before autoradiography.

Areas of increased FMISO binding were assessed using an objective thresholding method (see below) by an assessor blind to the results of the histological analysis. Autoradiographic images were smoothed using a 15×15 smoothing kernel. The relative optical density (ROD) of the contralateral hemisphere was used to calculate the hypoxic threshold of the ischemic hemisphere, with intensities greater than the mean ± 4 SDs of the contralateral hemisphere defined as hypoxic. This was performed on a slide-by-slide basis. The area and ROD of regions above this threshold were measured for each tissue section and volumes of hypoxic binding calculated. The mean RODs of these areas and of the contralateral hemispheres were used to calculate a hypoxic:contralateral ratio. Validation was performed on a random sample of brains from different experimental protocols to ensure that the threshold was consistent with the manually outlined areas of increased binding and that the threshold did not detect binding within the nonischemic hemisphere.

Experimental Protocols
Initially, experiments were performed replicating the timing of previous [18F]FMISO experiments6 (Figure 1; cohort 1). [3H]FMISO was administered at 30 minutes (MCAo=0 hours), animals were reperfused at 2 hours, and perfusion fixation performed at 2.5 hours (n=6). The aim was then to establish that [3H]FMISO binding was sufficiently prolonged to allow autoradiography to be performed on animals kept alive for 24 hours after FMISO administration. To ensure that this delayed autoradiography was robust, a short duration MCAo (resulting in relatively weak signal) was used. Furthermore, comparisons were made to demonstrate that the delay in tissue
processing did not significantly alter the measured area or relative intensity of autoradiographic signal. All animals received FMISO at 30 minutes and were reperfused 45 minutes later (75 minutes from MCAo). One cohort underwent perfusion fixation at 24 hours (Figure 1; cohort 3; n=6), whereas a comparison group (cohort 2; n=6) was euthanized at 2.5 hours. The final experiments sought to confirm that FMISO binding is specific to hypoxia and does not occur in injured but well-reperfused tissues. One-hour MCAo was performed, and FMISO was administered 1 hour after reperfusion (cohort 4; n=9). Tissue fixation was performed at 24 hours to allow histological confirmation of tissue injury.

Results

Oxygen saturations were maintained ≥93% in all animals with the exception of 1 animal in cohort 4 (discussed below). There was a rapid decrement in laser Doppler flow at MCAo in all animals, with an average drop of 56±13% (mean±SD). The feasibility of [3H]FMISO autoradiography was first established by replicating the paradigm of previous [18F]FMISO experiments (cohort 1). The prolonged nature of [3H]FMISO binding was then confirmed, with areas of increased FMISO uptake still seen after 24 hours (cohort 3), despite the relatively short (45 minutes) hypoxic exposure interval (Figure 2). There were lower RODs in the hypoxic areas of the animals kept alive for 24 hours compared with the 2.5-hour cohort (same administered dose and exposure time); however, because of the more complete washout of tracer from the nonhypoxic areas, the hypoxic ratio (ROD of hypoxic area/ROD of contralateral hemisphere) was not significantly different (1.88±0.06 at 2.5 hours; 2.02±0.11 at 24 hours; NS; Figure 3). Similarly, there was no difference in the volume of hypoxic binding (25±6 mm³ at 2.5 hours; 28±5 mm³ at 24 hours).

In the final experiment to demonstrate that binding does not occur in reperfused tissue, 1 animal was excluded from analysis because the laser probe holder became dislodged before reperfusion. The remaining 8 animals showed reperfusion >75% of baseline. One of these animals was noted to have reduced SaO₂ on pulse oximetry (91%), and this animal showed a small area of FMISO binding (6.5 mm³; single outlier; Figure 4). All remaining animals showed minimal to no binding (<2 mm³). Clear-cut evidence of infarction was variable on analysis of the H&E sections. However, areas of neuronal loss were seen in all animals, even those with no frank infarction and no area of increased FMISO uptake (Figure 5).

The reason for the reduced SaO₂ in 1 animal remains obscure. The animal showed good ventilatory effort, and the SaO₂ was unresponsive to increasing the oxygen:air ratio. When the SaO₂ probe was checked subsequently, it was found...

![Figure 2](image-url)

**Figure 2.** Representative autoradiographs and tissue sections showing the effect of prolonged reperfusion interval on FMISO binding. Column 1 indicates 2.5-hour reperfusion; column 2, 24-hour reperfusion. A and B, Raw autoradiographs. C and D, Smoothed and thresholded autoradiographs (area of increased [3H]FMISO binding highlighted in red). E and F, Photograph of H&E-stained sections from which autoradiographs were obtained. As can be seen, after 24-hour reperfusion, the autoradiographic signal is largely preserved in the previously hypoxic region; however, there is complete washout of tracer from nonhypoxic areas. The histological changes of infarction are not apparent 2.5 hours after MCAo onset, whereas they have become apparent after 24-hour reperfusion (bar=1 mm).

![Figure 3](image-url)

**Figure 3.** Effect of prolonging the reperfusion interval on relative intensity and volume of FMISO binding. Prolonging the reperfusion interval from 75 minutes to 24 hours, without altering duration of MCAo or time of [3H]FMISO administration did not significantly alter either intensity (A) or volume (B) of [3H]FMISO binding (intensity is relative to contralateral hemisphere, and volume was measured semiautomatically based on a threshold of the mean +4 SD of the contralateral hemisphere).
to be operating normally. Study of the tissue sections from this animal revealed erythrocyte-filled vessels within the FMISO-bound territory (amygdala). Detailed study of the remainder of the MCA territory (which showed no FMISO binding) and the contralateral hemisphere revealed no other erythrocyte-filled vessels.

Discussion
The results presented have shown that [3H]FMISO may be used to image hypoxic tissue in animal models of ischemic stroke. Second, delaying tissue processing to allow the evolution of histological change does not significantly alter the detection of hypoxic areas, even in the case of a short ischemic interval with subsequent reperfusion. Finally, we have shown that [3H]FMISO binding does not generally occur after successful reperfusion.

These data have particular relevance for experiments to improve our understanding of the role of hypoxia in the evolution of ischemic injury after stroke and for interpretation of data obtained from human [18F]FMISO PET studies of stroke patients. Animal studies of outcome after hypoxia have been hindered previously by the problem that autoradiographic techniques require animal death, and therefore, there is not sufficient time for histological changes to develop. Imaging techniques such as MRI or small animal PET remove the need to euthanize the animal early; however, resolution is limiting in the commonly used rodent models. Oxygen probes have been used; however, there is then the difficulty of only sampling a small brain region. Previous hypoxia autoradiography studies using iodine-125 iodoazomycin arabinoside in a rat permanent MCAo model used 7-hour histology and showed binding in an area equivalent to the area of injury at
this early time point.\textsuperscript{11,12} Iodine-125 iodoazomycin arabinoside was administered 2 hours after onset of MCAo, which although a realistic time frame for human studies, may be toward the end of the evolution of stroke in the rat model.\textsuperscript{13} We administered [\textsuperscript{3}H]FMISO at 30 minutes because at this time, much of the ischemic area remains potentially salvageable, yet tissue hypoxia is fully developed. Because of the large differences from our study (different tracers, times of administration and animal death, and use of permanent versus transient occlusion), it is difficult to make meaningful comparisons with these studies.

There has previously been demonstration of excellent agreement between [\textsuperscript{3}H]FMISO and [\textsuperscript{18}F]FMISO binding (slope = 1.01; \(R^2 = 0.98)).\textsuperscript{2} consistent with the negligible difference in chemical structures. [\textsuperscript{3}H]FMISO has many advantages for autoradiographic studies; however, it has not been used previously to image hypoxia in stroke. Additionally, there have not been previous attempts to delay autoradiography to allow development of histological changes. We demonstrated that this is possible without significant alteration in the hypoxic ratio or the area of binding, allowing direct comparison of hypoxic binding with histological outcome from the same tissue sections. Not only does this increase the power of the method to detect smaller changes in volumes of hypoxic tissue or infarct, it also allows direct comparison of their relative spatial distributions. Importantly, 24-hour autoradiography was effective even in the presence of comparatively low signal (45-minute interval for hypoxic binding to occur). FMISO binding is influenced by the degree of hypoxia and time.\textsuperscript{1} Therefore, as expected, shorter duration occlusion resulted in a smaller area of FMISO binding (cohorts 2 and 3; Figure 4). Importantly, prolonging the reperfusion interval alone did not significantly affect the bound volume (3 and 4). The lack of edema correction (not possible on autoradiographs) is unlikely to have significantly influenced this result because even in the 24-hour animals, the largest between-hemisphere volume difference from histological sections was \(<4\%\).

Human [\textsuperscript{18}F]FMISO studies have been revealing about the temporal and spatial evolution of hypoxia in stroke and in correlating these changes with clinical outcome. However, there are some unresolved questions that are not easily answered in human studies. The first of these, we sought to address in this study. Is binding specific to hypoxic tissue, or will there be ongoing binding after reperfusion (either spontaneous or after thrombolytic therapy)? Aside from 1 anomalous result, the answer seems to be that after effective reperfusion, there is no ongoing binding. Effectiveness of the original occlusion was confirmed by both laser Doppler monitoring and the presence of infarction or selective neuronal necrosis. The implication for human studies is that if patients effectively reperfuse before study, they will have a negative [\textsuperscript{18}F]FMISO PET scan. Regarding the single anomalous animal, there are a few possible explanations. The most plausible, and best backed by evidence, is that there was a variable degree of reperfusion within the MCA tree, and that superficial branches supplying the territory underlying the laser Doppler probe were well reperfused, whereas some of the deeper branches or small vessels were not. In support of this, the hypoxic binding in this animal was mostly within amygdala, and erythrocyte-filled vessels were only seen within this deep territory (despite apparently effective saline perfusion washout as judged by the lack of erythrocyte-filled vessels previously). Erythrocyte and fibrin retention within vessels in ischemic areas is well described (the "no reflow" phenomenon).\textsuperscript{14,15} The FMISO binding may have been further facilitated by reduced Sao2. Further indirect evidence that adequate reperfusion does prevent subsequent FMISO binding is that lengthening the interval from [\textsuperscript{3}H]FMISO administration to reperfusion increases the amount of binding seen.\textsuperscript{16} These results raise a discrepancy with previous work from our laboratory showing [\textsuperscript{18}F]FMISO binding after reperfusion in some animals.\textsuperscript{6} In retrospect, we believe this binding to have been attributable to ongoing ischemia from vasospasm/subarachnoid hemorrhage, resulting from vascular injury caused by poly-L-lysine–coated sutures.\textsuperscript{9}

**Summary**

We demonstrated the utility of [\textsuperscript{3}H]FMISO in delayed autoradiographic imaging of an animal stroke model, with direct correlation of histology from the same tissue sections. Furthermore, we have shown that binding does not occur in well-reperfused tissues, important confirmation for existing and ongoing [\textsuperscript{18}F]FMISO PET studies in human stroke. This technique has the potential to greatly improve our understanding of the role of cellular hypoxia in stroke because it provides a high-resolution spatial map of hypoxia in the whole brain.

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