Fluoromisonidazole
A Metabolic Marker of Myocyte Hypoxia


Fluoromisonidazole is metabolically trapped in viable hypoxic cells in vitro. This property is the basis for the hypothesis that $^{18}$F-fluoromisonidazole can be used to detect hypoxic tissues noninvasively using positron emission tomography. To assess the potential usefulness of this compound as a marker for hypoxic myocardium, we measured the accumulation of $[^3]$H-fluoromisonidazole in isolated adult rat myocytes under normoxic, hypoxic (5,000 ppm O$_2$), and anoxic conditions. Both anoxia and hypoxia caused a marked increase in $[^3]$H-fluoromisonidazole accumulation. Relative to uptake during normoxia, uptake during anoxia was increased by 8.4-fold at 60 minutes and 26.5-fold at 180 minutes ($p<0.001$). During hypoxia, uptake was increased by 4.4-fold at 60 minutes and by 15.3-fold at 180 minutes ($p<0.0001$) and occurred in the absence of significant cell injury as measured by release of creatine kinase and changes in cell morphology. Additional studies demonstrated a slow oxygen-insensitive efflux of fluoromisonidazole or labeled metabolite(s) from myocytes reincubated in drug-free medium. We conclude that fluoromisonidazole is avidly retained in hypoxic myocytes and thus may be suitable for noninvasive detection of hypoxic myocardium using positron emission tomography. (Circulation Research 1990;67:240–244)

An appropriate oxygen tension is a basic requirement for normal myocardial function and metabolism. However, little is known regarding myocardial cell Po$_2$ in humans, due primarily to methodological difficulties. Experimental techniques such as polarographic electrodes, microspectrophotometry, or coronary venous Po$_2$ sampling are invasive and thus not suitable for routine clinical use. Therefore, a noninvasive method for assessing tissue oxygenation has a major advantage.

Studies have shown that misonidazole, a drug that exhibits electron affinity, and the congenor fluoromisonidazole are metabolically trapped in viable cells at reduced Po$_2$. This property is the basis for recent interest in using radiolabeled fluoromisonidazole to image hypoxic tissues. This technique may be particularly valuable when applied to the heart, because detection of viable hypoxic myocardium is an important clinical problem. Initial in vivo studies have demonstrated increased deposition of $[^3]$H-fluoromisonidazole in experimental myocardial ischemia. $[^18]$F-Fluoromisonidazole has been successfully synthesized, and preliminary studies using positron emission tomography (PET) in dogs have shown enhanced regional deposition of this tracer in ischemic myocardium. $^7$

To study the relation between myocyte Po$_2$ and fluoromisonidazole uptake, we measured the uptake and retention of $[^3]$H-fluoromisonidazole in isolated adult rat myocytes, because this model allows more precise control of cellular Po$_2$ than can realistically be achieved in vivo. The results indicate a direct relation between reduced cellular Po$_2$ and enhanced fluoromisonidazole uptake in cardiac myocytes.

Materials and Methods

Isolated, calcium-tolerant adult rat myocytes were obtained by collagenase dissociation. $^{10}$ Hearts were rapidly excised, mounted on a Langendorff apparatus, and perfused for 1 minute at constant pressure (85 mm Hg) with calcium-free modified minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with insulin, taurine, and creatine. All buffers were prewarmed to 37°C, equilibrated with 95% O$_2$–5% CO$_2$ at pH 7.4, and adjusted for osmolarity. After the initial perfusion cleared blood from the heart, perfusion continued for another 15 minutes with medium containing 0.06%
crude collagenase and 0.03 mM calcium. The hearts were then removed from the cannula and minced with razor blades; individual cells were released by gentle pipetting and collected by centrifuging the suspension at 350 rpm for 2 minutes. The pellets were washed with collagenase-free medium, and calcium chloride was added gradually until a concentration of 1 mM was achieved. After being allowed to equilibrate for 45 minutes, the final pellet was resuspended, a cell count was performed, and the cells were diluted to approximately 200,000 cells/ml. Contour Permanox tissue culture dishes (Miles Scientific, Naperville, Ill.), precoated with laminin solution (8 

µg/ml), were plated with 1.25 ml of the cell suspension. Placement of a plastic ring on the plates before plating allowed attachment of cells only in the center of the dish. This was done to minimize differences in the depth of the medium covering the cells due to meniscus effects at the periphery. The medium was replaced with fresh medium containing 100 mg/ml bovine serum albumin, and the cells were kept overnight at 37°C in 95% room air-5% CO₂. Studies were performed 24 hours after isolation.

After overnight incubation, the cells were rinsed and covered with a thin layer of medium (1 ml per 60-mm dish) containing [³H]fluoromisonidazole (50 

µM). The drug was synthesized as previously described10 and stored in a dilute solution in absolute ethanol at 5°C to minimize radiolytic decomposition; its radiochemical purity by high-performance liquid chromatography was greater than 99%. The cells were exposed to the desired oxygen conditions using the thin film culture techniques of Koch.12 The dishes were placed in airtight aluminum chambers connected to a manifold system that allowed serial evacuation at reduced pressure and flooded the chambers with a specified premixed gas mixture. Evacuation at reduced pressure causes rapid outgassing of residual cellular oxygen, and the thin medium layer allows rapid equilibration with the replacement gas mixture. To scavenge any small amounts of oxygen, a separate dish containing sodium dithionate was added to each anoxic chamber. Anoxia was verified at the end of incubation by noting the maintenance of the reducing capacity of the sodium dithionate solution, namely, the ability to rapidly decolorize methylene blue at the end of each experiment. Independent studies12 have shown that this procedure does not damage cells. Each chamber was equilibrated with one of the following mixtures: 1) 95% room air-5% CO₂ (normoxic), 2) 5,000 ppm O₂-5% CO₂-balance N₂ (hypoxic), or 3) 5% CO₂-95% N₂ (anoxic); the chamber was kept at 37°C for specified times up to 180 minutes.

An initial set of experiments was performed to determine the time course of [³H]fluoromisonidazole uptake. Cells were incubated under normoxic or anoxic conditions for various time periods between 0 and 180 minutes. At the end of the experiment, the medium was removed, and the cells were rinsed twice with cold albumin-free medium to remove unbound labeled drug and fixed with 70% ethanol. The pH of the supernatant at the end of the experiment averaged 7.32 and was not different between hypoxic (pH 7.33, n=6) and normoxic (pH 7.31, n=5) conditions. Cell numbers per dish were determined by counting 13 premarked high-power fields and correcting for the cell attachment area. The plates were scraped, and the cell material was placed in scintillation vials along with the scintillation cocktail (Instagel, Packard Instrument Co., Inc., Downers Grove, Ill.) for liquid scintillation counting. Counting efficiency was determined by the external standards ratio, and the drug uptake data are expressed as picomoles per 10⁶ cells. Cell-free dishes were included in each experiment to account for background due to nonspecific adherence of the drug to the laminin. Several plates of cells exposed to 180 minutes of anoxia were fixed and embedded in epoxy; ultrathin sections were prepared for examination of cell morphology by electron microscopy.

To assess fluoromisonidazole uptake during less severe hypoxia and to relate uptake to indicators of cell injury, cells were incubated under normoxic, hypoxic (5,000 ppm O₂-3.5 mm Hg), or anoxic conditions for 60 or 180 minutes. At the designated times, the dishes were removed, and the medium was collected and immediately centrifuged and stored at 0°C for subsequent determination of creatine kinase (CK) activity. CK activity was measured spectrophotometrically and expressed as units per 10⁶ cells. Total cell counts were determined as before. In addition, the percentage of myocytes retaining normal rodlike morphology was determined, providing an additional indicator of cell injury. Tritium activity was determined as before.

Additional experiments were performed to study whether metabolic trapping of fluoromisonidazole is reversible. Cells were prelabeled with [³H]fluoromisonidazole under anoxic conditions for 2 hours as before, and then they were rinsed four times and covered with drug-free medium. One group of dishes was processed immediately to determine tritium content of both cells and medium. Two additional groups were reincubated for an additional 3 hours under either normoxic or anoxic conditions. Cell number and percent rods were determined, and cell and medium samples were counted for tritium as before. In four dishes, 0.1 ml medium was removed for scintillation counting and replaced with 0.1 ml cold medium at 20-minute intervals during the 3-hour incubation to determine the time course of efflux of radioactivity from the cells. Differences in percent rods, CK release, and [³H]fluoromisonidazole uptake between experimental groups were evaluated using the Kruskal-Wallis test.

**Results**

The isolation and plating procedures yielded 22.9×10⁶±6.2×10⁶ calcium-tolerant cells/dish, of which 85% retained the normal rod-shaped morphology. As opposed to rounded cells, rods exhibited
was significantly greater at 60 minutes and continued to increase up to 180 minutes. The mean uptake rate under anoxic conditions was 12.96 pmol/10^6 cells/min.

Table 1 shows data from the second series of experiments in which fluoromisonidazole uptake was compared with changes in cell morphology and CK release. Hypoxia (5,000 ppm O_2) caused a 4.4-fold increase in uptake versus normoxic controls at 60 minutes (p<0.0003) and a 15.3-fold increase at 180 minutes (p<0.0001). Hypoxia did not decrease the percentage of rod-shaped myocytes nor cause an increase in CK release. Anoxia resulted in an 8.4-fold increase in [3H]fluoromisonidazole at 60 minutes (p<0.001) and a 26.5-fold increase at 180 minutes (p<0.001). Under anoxic conditions, there was a nonstatistically significant decline in the percentage of rods at 60 minutes from 81±4% under control conditions to 71±13% and a small increase in CK release from 0.059±0.011 IU/10^6 cells under control conditions to 0.095±0.12 IU/10^6 cells (p=0.048). By 180 minutes, evidence of anoxic damage was more striking, with a reduction in rods to 53±16% and an increase in CK release to 0.218±0.028 IU/10^6 cells.

Figure 2 shows the results of experiments in which cells prelabeled with [3H]fluoromisonidazole were reincubated in drug-free medium for 3 hours under either normoxic or anoxic conditions. Compared with cells assayed immediately following the prelabeling and rinsing procedures, there was a reduction in cell-associated radioactivity in cells reincubated under either condition and a corresponding rise in tritium activity in the medium. This finding indicates loss of tritium activity from the cells. The percent reduction in activity in cells reincubated under normoxic conditions was not different from those reincubated under anoxic conditions (24.9% versus 27.8%, p=NS). Figure 3 shows the time course of release of drug from reoxygenated cells; the data were obtained from four cell plates in which samples of medium were collected every 20 minutes during the reincubation period.

**Discussion**

PET imaging of [18F]fluoromisonidazole has been proposed as a technique for identifying viable hypoxic

**TABLE 1. Effects of O_2 on Cell Morphology, Creatine Kinase Release, and Uptake of [3H]Fluoromisonidazole**

<table>
<thead>
<tr>
<th>Condition</th>
<th>O_2 concentration (ppm)</th>
<th>n</th>
<th>Rods (%)</th>
<th>CK release (IU/10^6 cells)</th>
<th>[3H]FMISO (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>At 60 minutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>210,000</td>
<td>13</td>
<td>81±4</td>
<td>0.059±0.011</td>
<td>56±16</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>5,000</td>
<td>13</td>
<td>81±4</td>
<td>0.065±0.014</td>
<td>249±35*</td>
</tr>
<tr>
<td>Anoxic</td>
<td>0</td>
<td>10</td>
<td>71±13</td>
<td>0.095±0.012</td>
<td>471±93*</td>
</tr>
<tr>
<td><strong>At 180 minutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>210,000</td>
<td>13</td>
<td>78±4</td>
<td>0.094±0.019</td>
<td>73±20</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>5,000</td>
<td>13</td>
<td>78±4</td>
<td>0.069±0.009</td>
<td>1,117±175*</td>
</tr>
<tr>
<td>Anoxic</td>
<td>0</td>
<td>10</td>
<td>53±16</td>
<td>0.218±0.028</td>
<td>1,922±292*</td>
</tr>
</tbody>
</table>

Values are mean±SD. CK, creatine kinase; FMISO, fluoromisonidazole; n, number of cell dishes.

*p<0.05 compared with normoxic controls.
tissue in tumors and in nonmalignant tissues such as heart and brain. A direct relation between enhanced fluoromisonidazole uptake and reduced cellular PO2 has been previously shown in tumor cell lines in vitro. The current study is the first to demonstrate this relation in cardiac myocytes.

In the absence of oxygen, fluoromisonidazole uptake was increased approximately 25-fold after 180 minutes. Although uptake was continuous throughout the study, the rate of accumulation is nonlinear. The explanation for the nonlinear uptake is uncertain. One possibility is that cellular PO2 was not constant during the experiment. For example, despite the degassing procedure and the use of sodium dithionite, absolute intracellular anoxia may not have been achieved, and the small amount of residual oxygen may have been gradually used up by the cells. This may explain the apparent sharp increase in drug accumulation at 120–180 minutes as well as the increase in percentage of rounded cells, which occurs between 60 and 180 minutes (see Table 1).

These experiments clearly demonstrate a general relation between fluoromisonidazole uptake and the severity of hypoxia. However, an exact relation between drug uptake and cellular PO2 cannot be defined. The procedures used ensure rapid oxygen equilibration between the gas phase in the aluminum chambers and the thin layer of medium overlying the cells. However, it is probable that there are small oxygen gradients between the unstirred layer of medium immediately adjacent to the cells and the cell interior where drug trapping occurs. Thus, although the presence of 5,000 ppm O2 in the chamber inhibited binding by approximately 50%, the actual cellular PO2 was in all likelihood somewhat less.

The exact biochemical mechanism responsible for trapping of fluoromisonidazole in hypoxic heart cells is uncertain. A likely mechanism involves formation of reduced metabolites, including radical intermediates, via the action of intracellular nitroreductases. It is hypothesized that one or more metabolites are trapped either by binding to intracellular macromolecules or by virtue of having a lower cell membrane permeability than the parent compound. Oxygen is thought to inhibit this process via oxidation of the one-electron reduction product with reformation of the parent drug in a "futile" cycle.

These experiments also demonstrate an oxygen-insensitive efflux of fluoromisonidazole and/or labeled metabolites from the myocytes. Thus, net uptake of this drug reflects both an oxygen-sensitive trapping mechanism and a release process that is relatively independent of PO2. In these studies, the rate of release was approximately 15% of the net uptake rate under anoxic conditions. Although this should not impose serious limitations on the use of this drug to image hypoxic myocardium, further studies are needed to define the relative magnitude of both uptake and release in vivo.

The data implicate cellular hypoxia as the principal cause for enhanced fluoromisonidazole deposition in ischemic myocardium. Ischemia is characterized not only by reduced tissue PO2 but also by excessive local accumulations of metabolic end products, and pH may fall to 6.0. However, in the current studies, the incubation medium can be considered a limitless "sink" that provides a constant supply of oxygen and other metabolic substrates and prevents excessive proton accumulation. This is evidenced by a fall in pH to only 7.32. Therefore, it is unlikely that severely ischemic conditions are a prerequisite for binding. This conclusion is in agreement with the studies of Shelton et al, who reported increased fluoromisonidazole extraction in isolated rabbit hearts perfused at normal flow rates with a hypoxic buffer.

**Figure 2.** Bar graphs showing release of radioactivity from cells that were prelabeled with [3H]fluoromisonidazole, rinsed, and covered with drug-free medium. Compared with cells assayed immediately after rinsing (no reincubation), there was a loss of radioactivity from cells reincubated for 3 hours under either normoxic or anoxic conditions, with a corresponding increase in the radioactivity in the medium. Loss of radioactivity was not different between cells reincubated under normoxic conditions versus those reincubated under anoxic conditions.

**Figure 3.** Graph showing the time course of release of tritium-labeled fluoromisonidazole ([3H]FMISO) or metabolites from prelabeled cells reincubated for 3 hours in drug-free medium under normoxic conditions. The data shown are mean±SD for four cell plates. Loss of radioactivity occurs gradually after reexposure to oxygen. The data are optimally fit by the second-order expression y=100–0.19x + 0.0004x2, r=0.998.
solution, and with our own in vivo studies showing increased fluoromisonidazole deposition in regions of myocardium that were only mildly underperfused, with flows greater than 60% of normal.8

Another conclusion from these studies is that enhanced drug uptake can occur, at least in this model, at sublethal levels of hypoxia. The moderate hypoxia induced by incubation with 5,000 ppm O2 resulted in no changes in cell viability, as measured by loss of rod-shaped morphology, and no significant release of CK. In previous studies, cytotoxic enzyme release has been shown to precede the development of irreversible cell injury.18 The absence of contraction band necrosis by electron microscopy is further evidence that the hypoxic injury in our studies was reversible. Enhanced uptake of fluoromisonidazole in the absence of irreversible cell injury supports the hypothesis that PET imaging of [18F]fluoromisonida- zole will provide a positive image of hypoxic but viable myocardium.

The data have several additional implications regarding the use of [18F]fluoromisonidazole for hypoxia imaging. First, fluoromisonidazole uptake is rapid relative to the radioactive half-life of fluorine 18 (1.8 hours). Second, the uptake rate reflects not only the presence of hypoxia but also its severity. The latter supports the notion that PET imaging of [18F]fluoromisonidazole will provide quantitative information regarding regional myocardial oxygenation. Third, since net accumulation of this tracer reflects both oxygen-sensitive uptake and oxygen-insensitive efflux, modeling approaches to analysis of imaging data will need to consider both processes.

In conclusion, hypoxia-dependent uptake of the 2-nitroimidazole fluoromisonidazole is the basis for a unique strategy for detecting cellular hypoxia and thus may provide important information regarding myocardial metabolism. As an experimental tool, it may be useful for studying the physiology of oxygen delivery and utilization in the heart. PET imaging of [18F]fluoromisonidazole may allow clinical application of this compound. Noninvasive detection of viable hypoxic myocardium may be particularly useful in selecting patients for revascularization procedures.

References


Key Words • fluoromisonidazole • hypoxia • myocytes • nitroimidazoles
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