Low Molecular Weight Iron in Cerebral Ischemic Acidosis In Vivo

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Background and Purpose—Iron-catalyzed radical generation is a potentially significant mechanism by which extensive tissue acidosis exacerbates brain injury during ischemia/reperfusion. We hypothesized that levels of low-molecular-weight (LMW) iron increase during in vivo global cerebral ischemia in a pH-dependent manner, potentially catalyzing oxidant injury. The present study quantified regional differences in LMW iron during global cerebral incomplete ischemia and determined whether augmenting the fall in ischemic tissue pH with hyperglycemia also amplifies free iron availability.

Methods—Dogs anesthetized with pentobarbital-fentanyl were treated with 30 minutes of global incomplete cerebral ischemia produced by intracranial pressure elevation. Cerebral energy metabolites (ATP, phosphocreatine) and intracellular pH (pHi) were measured by 31P magnetic resonance spectroscopy. Preischemic plasma glucose level was manipulated to titrate end-ischemic pHi. After ischemia, brains were perfused with cold phosphate-buffered saline solution; then 16 different brain areas were sampled, filtered to separate the LMW fraction (<30 000 D), and assayed by rapid colorimetric assay for tissue iron. Total iron, LMW iron, and protein in each sample were measured in sham-operated (no ischemia, n=8), normoglycemic ischemia (ISCH [glucose 7±4 mmol/L], n=7), and hyperglycemic (GLU-ISCH [glucose 31±3 mmol/L], n=9) groups.

Results—High-energy phosphates fell to near zero values in both ISCH and GLU-ISCH groups by 30 minutes but remained unchanged in the sham-operated group. As expected, pH decreased during ischemia but to a greater extent in GLU-ISCH (6.20±0.05 in ISCH, 6.08±0.04 in GLU-ISCH, P<.05). Iron could be detected in all areas of the brain in sham-operated animals, with the highest amounts obtained from subcortical areas such as the hippocampus, pons, midbrain, and medulla. Total iron was higher in ISCH relative to sham-operated animals and higher in cortex and pons relative to GLU-ISCH. Regional LMW (as a percentage of total iron; LMW/total iron) was elevated in numerous brain areas in ISCH, including cortical gray matter, cerebellum, hippocampus, caudate, and midbrain. LMW/total iron was higher in GLU-ISCH versus ISCH in cortical gray matter only. In other brain areas, ischemic LMW/total iron was equivalent in glucose-treated or normoglycemic animals (white matter, thalamus, pons, medulla) or lower in the glucose-treated group (cerebellum, hippocampus, caudate, midbrain).

Conclusions—These data demonstrate that levels of total and LMW iron increase with global cerebral ischemia in the majority of cortical and subcortical regions of normoglycemic brain. However, exacerbation of ischemic acidosis via glucose administration does not increase tissue iron and produces a greater increase in the LMW fraction in cortical gray matter only. In other brain regions, total and LMW iron availability is similar to that of nonischemic animals. (Stroke. 1998;29:487-493.)

Key Words: acidosis • iron • cerebral ischemia • spectroscopy, nuclear magnetic resonance

Iron-catalyzed oxygen radical production has long been hypothesized to play a prominent role in brain injury during ischemia/reperfusion. Via Fenton-Haber chemistry, ferrous iron (Fe2+) interacts with hydrogen peroxide or superoxide to produce hydroxyl radical and other free radicals that damage cell protein and DNA structure.1-3 Alternatively, iron is an effective catalyst of tyrosine nitration by another cytotoxic oxidant, peroxynitrite.4 Furthermore, radical-mediated mechanisms may be particularly relevant to cerebral injury when the ischemic insult is accompanied by extensive tissue acidosis, eg, during ischemia in hyperglycemic subjects. Rehncrona et al5 hypothesized that lactic acidosis increases iron dissociation from carrier proteins that use carbonate binding, whereas carbonic acidosis (eg, from tissue CO2) acts to stabilize bicarbon-
plays an important role in acidosis-mediated mechanisms of ischemic injury, the conditions of iron release, regional distribution of prooxidant iron, and participation in lipid peroxidation have not been clearly outlined.

We have previously shown that reducing intracellular pH (pHi) to <6.0 and bicarbonate ion to <1 to 2 mmol/L during hyperglycemic cerebral ischemia produces a marked secondary deterioration of brain ATP and cerebral blood flow during reperfusion.9 This rapid and profound secondary injury can be ameliorated with antioxidant treatments, including the iron chelator and superoxide scavenger, deferoxamine.10,11 Therefore, we wished to further examine the role of iron in ischemic injury and quantify the increase in nonprotein bound iron that ordinarily occurs during global cerebral ischemia in vivo. The goal of the present study was to evaluate regional distribution of cerebral iron during ischemic conditions compared with the nonischemic brain. Furthermore, we tested the hypothesis that reduction of tissue pH during hyperglycemic ischemia amplifies free iron, some component of which may participate in oxidant-mediated brain injury.

Materials and Methods

This study was approved by the institutional animal care and use committee and is in compliance with the guidelines of the National Institutes of Health for care and handling of animals. A well-characterized model of global incomplete cerebral ischemia in dogs was used.9,12 Male dogs (10 to 15 kg) were anesthetized with intravenous fentanyl (50 μg/kg) and pentobarbital (6 mg/kg, followed by 3 mg/kg per hour) and mechanically ventilated with supplemental oxygen. The dogs were paralyzed with pancuronium bromide (0.1 to 0.2 mg/kg). Systemic arterial and venous catheters were placed for blood pressure monitoring, blood gas sampling, and infusion of intravenous fluids and medications. After complete retraction of temporals muscles, a midline burr hole was placed near the junction of the coronal suture for placement of a superior sagittal sinus. A Silastic ventricular drain catheter (Cordis) was placed into the ventricle via a second burr hole for intracranial pressure monitoring and during ischemia. Baseline measurements of arterial and venous blood pressure and intracranial pressure were measured with Statham transducers. Serum glucose pressure were measured with Statham transducers. Serum glucose, and MRS spectra were obtained in all groups. To create global incomplete cerebral ischemia, warmed cerebrospinal fluid was infused into the lateral ventricle from a reservoir, raising intracranial pressure and lowering cerebral perfusion pressure to a controlled level of 10 to 15 mm Hg, yielding a low residual cerebral blood flow. The ischemic period was 30 minutes.9,13 MRS data were collected throughout the experimental period in one 6-minute epoch and three 8-minute epochs. To end ischemia, the reservoir was disconnected and intracranial pressure rapidly returned toward baseline. The animal was removed from the magnet, and a left lateral thoracotomy was performed with ventricular cannulation and perfusion with 3 L of phosphate-buffered 0.9% NaCl solution to clear blood from the cerebral vasculature. The skull cap was removed at 15 minutes of reperfusion in each animal, and 16 different brain areas were sampled and frozen in liquid nitrogen for biochemical analysis.

Brain samples were evaluated for levels of LMW and total iron by the rapid colorimetric method as described by Fish.14 All assay solutions were made from double-chelated water to minimize iron contaminants (Chelex-100, BioRad). All chemicals were purchased from Sigma Chemical Co. Samples were weighed and then homogenized in 1-mmol/L EDTA. An aliquot was obtained for subsequent analysis of total iron and protein. The LMW aliquot was centrifuged in a three-step process: (1) at 3000 rpm for 20 minutes, (2) at 19 000 rpm for 40 minutes, and (3) the supernatant was then spun at 6000 rpm for 60 minutes through a 30 000 molecular weight nylon filter collection system (Vanex VG8504–00, Vanguard International) to separate the LMW fraction. Total and LMW iron samples were assayed by incubating 400-μL aliquots with 200 μL 1.2 mol/L hydrochloric acid–0.285 mol/L potassium permanganate solution for 2 hours at 60°C to release tissue iron. Next, 40 μL of 2 mol/L ascorbic acid–5 mol/L ammonium acetate–6.5 mmol/L ferrozine–13.1 mmol/L neocuproine solution was added to chelate ferric iron species and other potentially interfering metals. To precipitate iron, 500 μL of 1 mol/L EDTA in 3% HCl was added with 50 μL of 2.5 mol/L ammonium acetate. After 5 minutes, 100 μL of 2.5 mol/L ammonium acetate was added, and the samples were allowed to sink to the bottom of the centrifuge tube. The iron precipitates, total iron samples were centrifuged for 1 minute on an Allied-Fisher Scientific Micro–Centrifuge model 235C. A standard curve was generated for the range of 0.01 to 2.0 μg/mL, and all samples were measured at 562 nm with a Milton Roy Spectronic 601 spectrophotometer. Total protein levels were determined using the Bradford method.

Because minimal differences were apparent in the eight cortical regions, the data are presented as summed gray matter and individually represented deep brain structures. Data are normalized to total iron within the sample and to the sample wet weight. To evaluate reproducibility and assess intra-animal variability, a brain region from one sham-operated, one ISCH, and one GLU-ISCH animal each was homogenized, and several samples from the same region per animal were assayed using study methods. The coefficient of variation for this repeated analysis was 3.6% <0.01%. To these known samples, predetermined amounts of iron were added. Measurements were accurate to within 0.06 μg iron.

Because cerebral blood flow was not measured in the present study, severity of ischemia was assessed by the lack of any residual phosphocreatine signal and severe depression of ATP by end-ischemia as previously observed with this ischemic model and duration.9,13 Therefore, any animal with measurable phosphocreatine (>5%) by 30 minutes of ischemia was excluded from the study. Eight animals were excluded on this basis (3 normoglycemic and 3 hyperglycemic dogs).
The data are presented as mean ± SEM, and significance was set at P < 0.05 for all tests. Physiological data were analyzed with a repeated measures ANOVA, with treatment as the between-subject factor and time as the within-subject factor. If group or time interactions were significant, a Newman-Keuls test was used to distinguish individual groups at specific time points. Neurochemical data were analyzed using a one-way ANOVA and Newman-Keuls test.

Results

Physiological data are contained in Table 1. Arterial blood gases were controlled to maintain constant oxygenation and arterial CO₂; however, arterial pH was depressed in both ischemic groups relative to sham-operated animals. Epidural temperature decreased in both ischemic groups compared with sham but in an equivalent manner. During ischemia, arterial blood pressure initially rose in ISCH and GLU-ISCH, then decreased to baseline levels. Cerebral perfusion pressure was maintained at approximately 10 mm Hg, and there were no differences between the ischemic groups. Cerebral perfusion pressure and intracranial pressure remained unchanged over time in the sham-operated group. Plasma glucose was elevated throughout ischemia in GLU-ISCH relative to both ISCH and sham groups. During ischemia, hemoglobin was lower than in the sham-operated and ISCH groups.

As previously shown in this model, high-energy phosphates declined sharply during the 30-minute period of intracranial pressure elevation (Fig 1). At end-ischemia, phosphocreatine and ATP levels were similar between ISCH and GLU-ISCH. As expected, pH declined in both ischemic groups but to a greater extent in GLU-ISCH (Fig 2). End-ischemic pH was 6.20 ± 0.05 in ISCH and 6.08 ± 0.04 in GLU-ISCH (P < 0.05).

Levels of total and LMW iron were determined in 16 different brain regions. Because minimal differences were apparent in the eight cortical regions, the data are presented as summed gray matter (Table 2). Iron could be detected in all areas of the brain in sham-operated animals, with the highest amounts obtained from subcortical areas such as the hippocampus, pons, midbrain, and medulla. Total iron was higher per gram of wet weight in ISCH than in the sham group in most brain areas and higher in ISCH than in GLU-ISCH in three brain regions. In gray matter, LMW iron was 3.06 ± 1.09 mg per gram of wet weight in ISCH animals than in the sham group.

### Table 1. Physiological Variables Before and During 30 Minutes of Cerebral Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>3</th>
<th>10</th>
<th>18</th>
<th>26</th>
</tr>
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<tbody>
<tr>
<td>pH</td>
<td>Sham</td>
<td>7.42 ± 0.04</td>
<td>7.42 ± 0.03</td>
<td>7.42 ± 0.03</td>
<td>7.42 ± 0.03</td>
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<td></td>
<td>ISCH</td>
<td>7.41 ± 0.03</td>
<td>7.43 ± 0.04</td>
<td>7.41 ± 0.03</td>
<td>7.36 ± 0.02*</td>
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<td>GLU-ISCH</td>
<td>7.43 ± 0.03</td>
<td>7.40 ± 0.04</td>
<td>7.33 ± 0.04†</td>
<td>7.31 ± 0.04†</td>
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<td>Paco₂, mm Hg</td>
<td>Sham</td>
<td>33.9 ± 3.7</td>
<td>33.9 ± 3.7</td>
<td>33.3 ± 2.7</td>
<td>33.8 ± 3.6</td>
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<tr>
<td></td>
<td>ISCH</td>
<td>36.1 ± 5.1</td>
<td>35.7 ± 5.2</td>
<td>33.7 ± 4.0</td>
<td>37.8 ± 6.0</td>
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<tr>
<td></td>
<td>GLU-ISCH</td>
<td>33.6 ± 0.4</td>
<td>34.9 ± 4.0</td>
<td>37.0 ± 3.7</td>
<td>35.2 ± 3.5</td>
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<tr>
<td>Paco₂, mm Hg</td>
<td>Sham</td>
<td>111.8 ± 14.1</td>
<td>112.2 ± 11.2</td>
<td>112.3 ± 16.1</td>
<td>113.4 ± 11.3</td>
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<tr>
<td></td>
<td>ISCH</td>
<td>126.3 ± 19.2</td>
<td>126.5 ± 15.5</td>
<td>116.6 ± 17.3</td>
<td>111.4 ± 16.3</td>
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<td></td>
<td>GLU-ISCH</td>
<td>135.0 ± 29.9</td>
<td>137.3 ± 29.7</td>
<td>125.4 ± 20.6</td>
<td>124.6 ± 18.9</td>
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<td>Epidural temp., °C</td>
<td>Sham</td>
<td>38.4 ± 0.6</td>
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<td>38.4 ± 0.6</td>
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<tr>
<td></td>
<td>ISCH</td>
<td>38.4 ± 0.7</td>
<td>38.3 ± 0.6</td>
<td>37.3 ± 0.6*</td>
<td>36.8 ± 0.5*</td>
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<td>GLU-ISCH</td>
<td>37.9 ± 1.0</td>
<td>37.7 ± 0.7</td>
<td>37.4 ± 0.7*</td>
<td>37.1 ± 0.7*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>Sham</td>
<td>141 ± 11</td>
<td>139 ± 13</td>
<td>142 ± 13</td>
<td>144 ± 14</td>
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<td></td>
<td>ISCH</td>
<td>135 ± 12</td>
<td>173 ± 30*</td>
<td>187 ± 29*</td>
<td>193 ± 34</td>
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<td></td>
<td>GLU-ISCH</td>
<td>138 ± 12</td>
<td>197 ± 39*</td>
<td>207 ± 41*</td>
<td>183 ± 54</td>
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<td>ICP, mm Hg</td>
<td>Sham</td>
<td>14 ± 5</td>
<td>15 ± 4</td>
<td>14 ± 5</td>
<td>16 ± 6</td>
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<td></td>
<td>ISCH</td>
<td>17 ± 3</td>
<td>162 ± 30*</td>
<td>175 ± 29*</td>
<td>124 ± 38*</td>
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<tr>
<td></td>
<td>GLU-ISCH</td>
<td>15 ± 5</td>
<td>185 ± 37*</td>
<td>197 ± 42*</td>
<td>173 ± 56†</td>
</tr>
<tr>
<td>CPP, mm Hg</td>
<td>Sham</td>
<td>126 ± 13</td>
<td>125 ± 13</td>
<td>127 ± 13</td>
<td>128 ± 15</td>
</tr>
<tr>
<td></td>
<td>ISCH</td>
<td>118 ± 11</td>
<td>11 ± 4*</td>
<td>12 ± 2*</td>
<td>10 ± 3*</td>
</tr>
<tr>
<td></td>
<td>GLU-ISCH</td>
<td>122 ± 13</td>
<td>12 ± 7*</td>
<td>10 ± 4*</td>
<td>10 ± 4*</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>Sham</td>
<td>3.4 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>3.9 ± 1.5</td>
<td>3.0 ± 0.5</td>
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<td>ISCH</td>
<td>4.7 ± 2.1</td>
<td>5.5 ± 3.3*</td>
<td>8.2 ± 5.0*</td>
<td>7.8 ± 3.8*</td>
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<td>GLU-ISCH</td>
<td>3.6 ± 0.4</td>
<td>30.6 ± 2.7†</td>
<td>32.5 ± 3.9†</td>
<td>32.2 ± 2.7†</td>
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<tr>
<td>Hemoglobin, g/dL</td>
<td>Sham</td>
<td>13.5 ± 1.6</td>
<td>13.8 ± 1.7</td>
<td>13.6 ± 1.6</td>
<td>13.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>ISCH</td>
<td>13.8 ± 2.0</td>
<td>15.2 ± 2.2</td>
<td>15.3 ± 2.0</td>
<td>14.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>GLU-ISCH</td>
<td>12.5 ± 1.6</td>
<td>11.3 ± 2.0*†</td>
<td>12.4 ± 2.1†</td>
<td>11.9 ± 0.1</td>
</tr>
</tbody>
</table>

pH indicates arterial blood pH values; MAP, mean arterial blood pressure; ICP, intracranial pressure; and CPP, cerebral perfusion pressure (MAP − ICP). Data are expressed as mean ± SEM. Groups: Sham, n = 8; normoglycemic ischemia (ISCH), n = 9; hyperglycemic ischemia (GLU-ISCH), n = 7.

*P < 0.05 from sham group; †P < 0.05 from ISCH group.
Figure 1. Phosphocreatine and ATP during 30 minutes of global cerebral ischemia. Data are expressed as percentage of baseline values (mean±SEM). Sham-operated, n=8. Hyperglycemic ischemia (GLU-ISCH), n=8. Normoglycemic ischemia (ISCH), n=7.

Figure 2. Intracellular pH during 30 minutes of cerebral ischemia. Data are mean±SEM. Sham-operated, n=8. Hyperglycemic ischemia (ISCH), n=7. Normoglycemic ischemia (ISCH), n=7.

mu/g wet wt in ISCH compared with 0.22±0.04 in sham-operated animals and 0.6±0.35 mu/g wet wt in GLU-ISCH. Similarly, white matter LMW iron was higher in ISCH relative to sham but not to GLU-ISCH (2.86±0.98, 0.36±0.25, and 0.15±0.05 mu/g wet wt in ISCH, GLU-ISCH, and sham, respectively).

Given the regional and intergroup variability in total iron, Table 2 presents the LMW values referenced to the total iron obtained from the region of interest. Regional LMW (percentage of total iron; LMW/total iron) was elevated in numerous brain areas in ISCH, including cortical gray matter, cerebellum, hippocampus, caudate, and midbrain. LMW/total iron was higher in ISCH versus ISCH in cortical gray matter only. In other brain areas, ischemic LMW/total iron was equivalent in glucose-treated or normoglycemic animals (white matter, thalamus, pons, medulla) or lower in the glucose-treated group (cerebellum, hippocampus, caudate, midbrain).

Discussion

These data demonstrate two important findings. First, measurable levels of total and LMW iron can be assayed throughout the brain, and these levels increase in a region-specific manner during normoglycemic ischemia. Elevated LMW iron relative to nonischemic animals was observed in both cortical and subcortical brain regions, most prominently in the cerebellum, midbrain, hippocampus, and cortical gray matter. Second, increased total or free iron was not uniformly observed in animals treated with hyperglycemic ischemia, which sustained the lowest end-ischemic pH. These findings raise fundamental questions regarding the mechanisms and timing of iron release during ischemic acidosis.

The role of iron in oxidant injury as a consequence of ischemia has been inferred from numerous studies that show benefit from treatment with strong iron chelators or from apotransferrin. We found previously that pretreatment with deferoxamine, but not conjugated deferoxamine or iron-saturated ferroxamine, ameliorates the secondary metabolic deterioration and severe hyperperfusion observed with ischemia complicated by extreme lactic acidosis. However, deferoxamine has significant radical scavenging properties in vitro via hydrogen-donating free hydroxamate groups, so its mechanism of action relative to iron chelation is not entirely clear. Oxidative injury has been ameliorated by inhibiting iron-dependent lipid peroxidation with aminosteroids after trauma and ischemia.

Consequently, whether ischemia-induced release of substantial amounts of reactive iron occurs in vivo is of interest. LMW ferrous iron has been demonstrated to increase at various reperfusion time points after cardiac arrest, with the large increases observed at 1 to 2 hours after arrest, and to be accompanied by increases in levels of lipid peroxidation. Our results with incomplete global cerebral ischemia are consistent with these earlier findings in cortical homogenates, and we now show that increased free iron availability is widespread in brain, including regions of the brain that are known to be highly vulnerable to ischemic insults. Measurable levels of LMW iron were present in the nonischemic brain, with greatest levels of iron in the white matter of all groups. Others have demonstrated iron concentration in white matter, midbrain, pons, and medulla. Our baseline nonischemic LMW iron values may be overestimated because of our fractionation procedure with serial centrifugations and because 1-mmol/L EDTA, a common chelating agent, was used in the homogenization procedure. However, major increases were apparent in postischemic tissue when compared with nonischemic samples subject to the same potential for homogenization and fractionation artifacts. The lower limit of resolution of the assay is 0.01 μg iron, and differences in iron seen in our study are well above the lower limit of the assay, supporting the validity of our findings.

A 30 000-D filter membrane was used to separate tissue homogenates; the fraction passing through the membrane was defined as LMW iron. This cutoff is comparable to that previously used to examine tissue harvested from ischemic animals. A 30 000-D filtering capacity allowed separation of delocalized iron from the biologically inactive form, ordinarily complexed to large proteins such as transferrin (80 000 D) and ferritin (440 000 D). We were specifically interested in iron release at this level because of the pH-dependent binding properties for transferrin and possibly ferritin, the latter of
which is a major source of total tissue iron. It seemed likely that pH-associated delocalization of iron would be through release and reuptake of iron from carrier/storage proteins. Nevertheless, our measurements do not determine the precise chemical identity or catalytic activity of the LMW iron; small iron-complexed moieties and heme-containing proteins less than 30 000 D are likely present as LMW species and potential nonparticipants in iron-catalyzed oxidative injury.

The mechanisms of iron release during ischemia are likely complex, potentially involving both intracellular stores and intravascular iron transport and delivery to brain. Because of the potential cytotoxicity of iron, physiological systems have developed intricate mechanisms to protect cells from oxidative injury. Brain iron is largely complexed to protein carriers (transferrin, lactoferrin) for intravascular and transmembrane transport and to ferritin for intracellular storage. Ischemic acidosis may drive iron dissociation from those carrier proteins with pH-dependent binding characteristics (eg, carbonate binding ion of transferrin).30 Once mobilized, free iron likely binds nonspecifically to a variety of small molecular moieties and augments the ordinarily small LMW nonprotein-bound tissue pool. In cortical homogenates, striking increases in LMW iron are observed at pH 6.0 when pH is reduced from 7.0 by direct addition of lactic acid. Furthermore, brain from decapitated hyperglycemic rats shows elevated LMW iron relative to normoglycemic controls.8

Surprisingly, we did not observe increased LMW iron in all brain regions in hyperglycemic ischemia despite reduction in pH, to values <6.0 in many glucose-treated animals. Total iron was higher in many but not all brain regions in ISCH; consequently, we evaluated regional LMW iron for each group as a percentage of total iron. LMW/total iron was elevated in hyperglycemic versus normoglycemic ischemia only in cortical gray matter. In other brain areas, ischemic LMW/total iron was equivalent or lower in brain of glucose-treated animals when compared with either normoglycemic or nonischemic brain, suggesting that lower end-ischemic pH does not exacerbate ischemia-induced iron availability for catalysis of oxidant injury mechanisms, at least within the one time-point window of our observations. We cannot exclude the possibility that lower pH in GLU-ISCH accelerated iron dissociation, peaking during ischemia or imme-

diate reperfusion with subsequent early elimination of the LMW tissue pool. In addition, the end-ischemic pH, achieved in ISCH and GLU-ISCH spanned a narrow range. We did not evaluate iron over a range of pH to determine an optimum pH associated with LMW iron augmentation or whether there is a threshold below which free iron begins to decrease because of other pH-related factors. Nevertheless, it seems unlikely that the large differences observed with ISCH versus GLU-ISCH are explained by these factors. A more plausible explanation is that the total iron pool, and consequently the LMW fraction, increases during ischemia/reperfusion in an unanticipated manner that is independent of pH drops. However, with hyperglycemic ischemia, total tissue iron and the LMW fraction are regionally heterogeneous and not uniformly higher than in nonischemic animals.

One hypothesis is that ischemic brain iron loads are linked to intravascular iron delivery and tissue transfer via normal or pathophysiological mechanisms. The ischemic insult, as determined by the near complete loss of ATP and phosphocreatine, was different between ischemic groups in plasma glucose level only (7 versus 31 mmol/L in ISCH and GLU-ISCH, respectively) and the severity of end-ischemic pH (6.20 versus 6.08). Our previous measurements with this model indicate that brain blood flow, as measured by radiolabeled microspheres, is reduced uniformly throughout the brain, with a residual flow of <10 to 12 mL/min per 100 g during both normoglycemic and hyperglycemic ischemia.9–12 However, we sampled tissue after 30 minutes of ischemia and 15 minutes of reperfusion in each animal. Postischemic hyperemia occurs in normoglycemic animals during early reperfusion9–12; hyperglycemic ischemia results in heterogeneous reperfusion15 and significant hypoperfusion.9–12,26,27 While it is uncertain that extensive hypoperfusion would account for the failure to see increased total and LMW iron in the GLU-ISCH group, de novo iron delivery from circulating iron containing proteins could be reduced during hyperglycemic reperfusion. Although the kinetics and mechanisms of iron transport across the posts ischemic blood-brain barrier are not known, iron transfer into cells via transferrin binding and endosomal internalization is thought to occur within minutes in healthy brain.5 If intravascular iron delivery to tissue represents a significant contribution

### TABLE 2. Iron Levels During Global Incomplete Ischemia

<table>
<thead>
<tr>
<th>Area</th>
<th>Total Iron/Weight, µg/g</th>
<th>LMW Iron/Total Iron, % of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n=8)</td>
<td>ISCH (n=9)</td>
</tr>
<tr>
<td>Gray matter</td>
<td>1.56±0.23</td>
<td>4.41±1.33†</td>
</tr>
<tr>
<td>White matter</td>
<td>0.55±0.14</td>
<td>2.92±0.95†</td>
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<tr>
<td>Cerebellum</td>
<td>0.09±0.03</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.40±0.04</td>
<td>1.17±0.31†</td>
</tr>
<tr>
<td>Caudate</td>
<td>0.52±0.06</td>
<td>0.98±0.25</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.71±0.14</td>
<td>2.58±0.99†</td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.32±0.07</td>
<td>0.83±0.26†</td>
</tr>
<tr>
<td>Pons</td>
<td>0.46±0.10</td>
<td>4.98±2.03†</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.25±0.11</td>
<td>0.99±0.32†</td>
</tr>
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</table>

All data are expressed as mean±SEM. Sham-operated (n=8); ISCH (n=9); GLU-ISCH (n=7).

*P<.05 from sham group; †P<.05 from GLU-ISCH.
to the dynamic pool in reperfused brain, then gross hyperperfusion would alter tissue iron content.

An alternative hypothesis is that the low levels of iron in GLU-ISCH are a function of the high plasma and tissue glucose levels per se. Hyperglycemia glycates many protein moieties, including transferrin27 and increases tissue autoxidation products. Transferrin-bound iron is low in rat hyperglycemic sera secondary to glycation, although the proteins remain redox-active, and target cell binding is depressed.28,29 Therefore, this source of iron dissociation may be inconsequential in GLU-ISCH versus ISCH. The effect of acute hyperglycemia on intracellular storage proteins such as ferritin is unknown; however, with intense oxidant stress, ferritin can be transformed into a hemosiderin-like protein,30 which is less reactive to Fe2+-releasing stimuli.31,32 The abnormal ferritin-Fe2+ complex would not be captured in our fractionation assay because of its relatively high molecular weight. Therefore, it is feasible that hyperglycemia alters carrier protein binding in normal or ischemic tissue and yields lower brain iron during reperfusion. Our present method for quantifying the total or LMW iron pool is not sufficiently sensitive to distinguish a baseline effect of hyperglycemia in sham-operated animals, independent of ischemia.

In conclusion, our findings strengthen the hypothesis that tissue iron and its potentially reactive LMW fraction are elevated after global incomplete cerebral ischemia and now demonstrate that the regional distribution is widespread. However, when the fall in tissue pH ordinarily observed with ischemia is exacerbated by systemic hyperglycemia, total iron and subsequently the LMW iron species available for catalysis of oxidant injury mechanisms are not elevated during early reperfusion. Whether other means of altering ischemic pH produce a similar lack of ischemia-induced iron release remains to be tested. However, we speculate that hyperglycemia alters ischemic tissue iron content in a pH-independent manner, possibly by decreasing iron delivery, tissue internalization, and binding protein function during severe but incomplete cerebral ischemia and early reperfusion.

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There is extensive evidence that hyperglycemia exacerbates cerebral ischemic injury. Whereas hyperglycemic patients have a worse neurological outcome after ischemic stroke, preischemic hyperglycemia aggravates ischemic brain damage in experimental animals. The mechanisms of this effect have not been elucidated. It has been suggested that the worsening of the brain damage is secondary to the greater pH reduction associated with hyperglycemic ischemia. More severe acidosis would in turn increase in the availability of free iron for formation of reactive oxygen species through the Haber-Weiss reaction. In the accompanying article, Lipscomb et al sought to test this hypothesis in vivo using a well-established canine model of temporary global cerebral ischemia. As anticipated, hyperglycemic ischemia produced a more severe reduction in brain pH than normoglycemic ischemia. Furthermore, normoglycemic ischemia elevated LMW iron in most brain regions. Unexpectedly, however, hyperglycemic ischemia enhanced this effect only in the neocortical gray matter and not in other brain regions. Rather, in most regions, ischemic hyperglycemia resulted in iron concentrations similar to those observed in normal brain. The data suggest that in this model, increased iron availability does not play a prominent role in the exacerbation of brain damage associated with hyperglycemic ischemia, with the possible exception of the cerebral cortex.

The regulation of iron homeostasis in the postischemic brain is poorly understood. Iron, a metal insoluble in water at physiological pH, is transported in the blood stream as Fe³⁺-bound transferrin (see Reference 4 for review). The transferrin-iron complex enters the cell by endocytosis via membrane-bound transferrin receptors. Once inside the cell, iron is sequestered by the storage protein ferritin from which it is released as Fe²⁺ (see Reference 5 for review). Expression of critical proteins involved in iron metabolism, eg, ferritin H and L chains, the transferrin receptor, and 5-aminolevulinate synthetase (the enzyme that synthesizes heme), is controlled by the iron response element–binding protein (IRE-BP). Thus, the intracellular concentration of catalytically active iron is tightly regulated by these proteins, the expression of which is controlled by IRE-BP. The effect of cerebral ischemia and/or hyperglycemia on this complex sequence of events remains to be elucidated. The work of Lipscomb et al is a step in the right direction because it provides much awaited in vivo data on the postischemic perturbation in iron homeostasis and on the effect of hyperglycemia on iron availability. This enlightening study constitutes a starting point for future investigations focusing on the effect of cerebral ischemia on the cellular and molecular factors regulating free intracellular iron.

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