Regional Cerebral Palmitate Incorporation Following Transient Bilateral Carotid Occlusion in Awake Gerbils

Osamu Tone, MD, Joseph C. Miller, PhD, Jane M. Bell, BA, and Stanley I. Rapoport, MD

[14C]Palmitate was injected intravenously in awake gerbils at various times after 5 minutes of bilateral carotid artery occlusion or a sham operation. Regional rates of incorporation of plasma palmitate into the hippocampus and other regions of the anterior circulation were determined relative to the mean rate of incorporation into regions of the posterior circulation using quantitative autoradiography and a ratio method of analysis. One day after bilateral carotid occlusion, relative palmitate incorporation was elevated significantly by 16% in the CA4 pyramidal cell layer and by 20% in the dentate gyrus of the hippocampus compared with sham-operated gerbils. At 3 days, significant elevations of this magnitude were found in the CA3 and CA4 cell layers, whereas relative incorporation was reduced by 26% in the CA1 pyramidal cell layer. At 7 days, the only significant difference from control was a 15% elevated incorporation in the CA3 pyramidal cell layer. Histologic examination indicated substantial cell death in the CA1 pyramidal layer at 3 days, with extensive glial reaction and phagocytic invasion at 7 days. Our results suggest that the turnover of palmitate-containing lipids is reduced in the CA1 layer of the gerbil hippocampus but that lipid synthesis is stimulated in hippocampal regions (CA3, CA4, dentate gyrus) affected by but recovering from transient bilateral carotid occlusion. (Stroke 1987;18:1120–1127)

Three types of responses have been reported in hippocampal subfields following 5 minutes of carotid artery occlusion in gerbils: delayed neuronal death of CA1 pyramidal cells, reactive change of CA2 pyramidal cells, and ischemic cell change in CA4 pyramidal cells.1-3 Cell losses are apparent in the CA1 region 3-4 days after 5 minutes of bilateral carotid occlusion in gerbils.1-3 The reactive and ischemic cell changes are reversible processes that are noticed first under the light microscope within 3-6 hours after carotid inclusion, are fully evident by 24 hours, but are absent by 21 days.2 The ischemic cell change in the CA1 region "matures" more slowly and is fully evident only after 2 days.

Several in vivo autoradiographic tracer techniques have been used to examine regional responses in the gerbil hippocampus to carotid occlusion. The regional cerebral metabolic rate for glucose (rCMRglc) measured with intravenously injected [14C]2-deoxy-D-glucose is elevated in the CA1 region 30 seconds and 10 minutes after recirculation following a 5-minute bilateral carotid artery occlusion, but rCMRglc is decreased at 2 days when spontaneous neuronal activity is absent.4 Brain protein synthesis, estimated from brain incorporation of intravenously injected [14C]leucine, is suppressed in the pyramidal cell layer and stratum radiatum of the CA1 hippocampal subfield 2 and 4 hours after a 5-minute bilateral carotid artery occlusion and is transiently reduced (at 2 but not at 4 hours) in CA2/CA3 pyramidal cells.5

In vivo brain lipid metabolism can be assessed using uniformly labelled [14C]palmitate to measure the rate of incorporation of unlabelled plasma palmitate into brain, defined as Jpelm.6-7 [14C]Palmitate is injected intravenously, and plasma specific activity of palmitate is followed for 4 hours, after which the animal is killed and regional brain radioactivity is determined by quantitative autoradiography. Jpelm equals the 4-hour brain radioactivity (mainly radioactive phospholipids) divided by the integrated plasma specific activity of palmitate and is related to the turnover and synthesis of brain lipids that contain palmitate.3

During development in rats, Jpelm is maximal at 20 days of age when myelin synthesis and brain growth are also maximal, and then Jpelm falls 4- to 10-fold to adulthood.10 Small reductions in Jpelm occur in the central auditory structures of rats following chronic unilateral cochlear damage; the reductions can be demonstrated to be significant with the use of a difference method to decrease the coefficient of variation inherent in absolute values of Jpelm.6,11,12 We thought it of interest to use the palmitate method to examine regional lipid metabolism in the gerbil hippocampus following bilateral occlusion of the common carotid arteries and to compare changes in metabolism, if any, with regional changes in cell morphology, rCMRglc, and local protein synthesis (see above). Because anesthesia alters plasma palmitate incorporation by brain13 and protects against ischemic injury,14,15 we used unanesthetized gerbils16 rather than anesthetized animals as in many previous studies.1,3,7-19 We also normalized 4-hour radioactivity in hippocampal regions to that in posterior circulation regions following [14C]palmitate injection so as to achieve low coefficients of variation for experimental data11,12 and to quantify any small experimental effects.
Materials and Methods

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 50–70 g were obtained from Tumblebrook Farms Inc. (West Brookfield, Mass.) and were fed a standard rabbit-guinea pig diet (NIH Production Animal Feed A, Agway Inc., St. Marys, Ohio) and water ad libitum. After a gerbil was anesthetized with 1–2% halothane, a 4-0 nylon suture was looped around each common carotid artery and passed through 2 silicon rings via a ventral midline incision (Figure 1). The ends of the suture were led out through the skin on each side of the gerbil's neck. Control gerbils were sham-operated by dissecting both common carotid arteries. Indwelling catheters containing 100 IU sodium heparin/ml isotonic saline were tied into a femoral artery to record blood pressure via a strain gauge transducer (Statham Instruments, Hato Rey, Puerto Rico) connected to a paper chart recorder (Model 2200, Gould Instruments, Cleveland, Ohio). One percent xylocaine was applied to all skin sutures.

After the procedure, the gerbil was restrained loosely in a plaster cast on a wooden block and was allowed to recover from anesthesia at an air temperature of 25–27°C. Four hours later, the suture ends were pulled tight for 5 minutes to occlude both carotid arteries. Use of a nylon suture and soft silicon rings minimized trauma to the common carotid artery, which occurs frequently with larger pneumatic or mechanical occlusion devices.19–22

Recirculation was initiated by loosening and removing the suture through a skin hole, leaving the silicon rings in place. In separate experiments, complete carotid occlusion was confirmed by measuring blood pressure in the external carotid artery when the suture was tightened. Mean arterial blood pressure and heart rate were measured via the femoral artery catheter before and during the 5-minute occlusion and for up to 50 minutes after recirculation. No gerbil convulsed during or following carotid occlusion.

The method of Kimes et al6 was used to compare incorporation of plasma [14C]palmitate into different brain regions. PE-50 polyethylene catheters filled with 100 IU/ml heparinized saline were inserted into the femoral artery and vein of a gerbil anesthetized with 1–2% halothane. Skin incisions were sutured and infiltrated with 1% xylocaine. The hindquarters of the gerbil were wrapped in a fast-setting plaster cast on a wooden block, and the gerbil was allowed to recover from anesthesia for 4 hours. Arterial and venous catheterizations were performed at the time of neck surgery in experimental gerbils that were to be injected with [14C]palmitate after 10 minutes or 1 hour of recirculation and in control gerbils. Gerbils also were studied between 1 and 7 days after carotid occlusion, in which case they were removed from their blocks after the occlusion, returned to their cages, and provided with food and water until they were reanesthetized and prepared as described above 4 hours before the injection of [14C]palmitate. Heart rate, mean arterial blood pressure, and hematoctrit were measured immediately before the injection of [14C]palmitate.

A solution of [14C]palmitate (uniformly labelled, 800–900 mCi/mmol, New England Nuclear, Boston, Mass.) in 5 mM HEPES buffer, pH 7.4, containing 5 mg/ml bovine serum albumin, was injected into the femoral vein at 450 μCi/kg 10 minutes to 7 days after the 5-minute occlusion or 4 hours after the sham operation in control gerbils. The gerbil was killed 4 hours after injection by an overdose of sodium pentobarbital and decapitated. The brain was removed, frozen in 2-methylbutane cooled to −65°C, and later sectioned at −20°C into 20-μm thick coronal sections. The sections were collected on glass slides, dried on a hot plate, and together with radioactive plastic standards (8–208 nCi/g) were exposed for 5 weeks to X-ray film (SB5, Kodak, Rochester, N.Y.). Adjacent dried sections were stained with cresyl violet for light histology. Brain regions were identified from a standard atlas of the gerbil brain.23

Autoradiographs were scanned with a microdensitometer (Model 700-10-90, Gamma Scientific, San Diego, Calif.)4 and were compared with adjacent stained sections to identify specific brain regions. Optical density was converted to regional brain radioactivity (rCwr) as dpm/g from the standard curve. Normalized rCwr was determined for 33 regions, including 21 regions supplied by the anterior cerebral circulation, by dividing rCwr by the average rCwr of 12 regions from the posterior circulation of the same gerbil:

\[
\frac{rC_{wr}}{\text{Avg posterior circulation } rC_{wr}} \times 100
\]

(1)

From the definition of \( J_{\text{sm}} \) (rCwr at 4 hours divided by integrated plasma specific activity of palmitate during 4 hours6) it can be seen that normalized rCwr in Equation 1 is identical to the rate of incorporation of plasma palmitate into the given brain region divided by the average rate of incorporation into brain regions of the posterior circulation, or the relative regional rate of...
incorporation of plasma palmitate. The integrated specific activity of plasma palmitate in both the numerator and denominator of Equation 1 is canceled out.

Comparison of mean normalized rCw between experimental and control gerbils indicates whether the regional rate of incorporation of plasma palmitate relative to incorporation in the posterior circulation regions is reduced or elevated. Equation 1 allows small experimental differences to be demonstrated statistically because ratios have smaller coefficients of variation than do absolute values of Jp.6,10

The time course of brain radioactivity following the intravenous injection of [14C]palmitate also was examined to see if, as in rats, 4-hour radioactivity represents a steady state.6 Forty-nine awake gerbils were injected with 450 μCi/kg [14C]palmitate, were killed 5 minutes to 48 hours later by an overdose of sodium pentobarbital, and then were decapitated. Whole blood samples also were taken. The brain was removed and dissected on ice into hippocampus and remaining tissue.25 Blood aliquots and weighed brain samples were solubilized at 50–70°C in 1–2 ml Soluene 350 (Packard Instruments, Downers Grove, Ill.); their radioactivity was determined by liquid scintillation spectroscopy (LS 9000, Beckman Instruments, Palo Alto, Calif.). Tissue radioactivity as dpm/mg tissue was corrected for intravascular radioactivity, which equals the product of the regional brain blood volume and blood radioactivity.26 Blood volumes were measured for the whole brain and the hippocampus using [14C]sucrose.26

In other experiments, gerbils were killed 1 hour to 7 days after a 5-minute bilateral common carotid occlusion or sham operation. After transaortic perfusion with 10 IU/ml heparinized saline followed by perfusion of 200 ml 3% (vol/vol) paraformaldehyde and 3% (vol/vol) glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, the brain was removed, stored overnight at 4°C in the fixative, embedded in plastic, cut into 3-μm sections, and stained with toluidine blue.

Analysis of variance and Dunnett’s multiple comparison test were used to compare experimental means with control means.27 The level of significance was p<0.01 rather than p<0.05 due to the multiple regions that were analyzed.28

Results

Mean arterial blood pressure (± SEM, n = 10) rose from 95 ± 3 mm Hg before bilateral carotid occlusion to 147 ± 3 mm Hg during occlusion (p<0.01). Blood pressure declined to 82 ± 4 mm Hg at 10 seconds after occlusion and returned to baseline by 50 minutes. Blood pressure before, during, and after occlusion was higher than that reported in gerbils anesthetized with pentobarbital, ether, or ketamine29-32 but did not differ from values reported in gerbils anesthetized with halothane or nitrous oxide.33 Mean heart rate rose from 381 ± 17 beats/min before occlusion to 469 ± 16 beats/min during occlusion (p<0.001) and returned to baseline after recirculation was initiated. In 67% of the gerbils, hypertension during occlusion was accompanied by an arrhythmia, usually a bradycardia. No significant difference (p>0.05) in body weight or hematocrit was noted between controls and gerbils injected with [14C]palmitate 10 minutes to 7 days after recirculation was established.

Figure 2 illustrates autoradiographs (A–D) and adjacent coronal cresyl violet-stained sections (E–H) through the dorsal hippocampus taken 4 hours after [14C]palmitate injection in a control gerbil and 1 hour, 3 days, and 7 days after bilateral carotid occlusion in experimental gerbils. No difference in optical density is apparent in the autoradiograph (Figure 2B) 1 hour after carotid occlusion compared with the control (Figure 2A). Optical densities in the CA1 layer was decreased 3 days after occlusion (Figure 2C) when optical densities in the CA3 and CA4 layers appeared elevated. The corresponding stained section (Figure 2G) showed pathology in many CA1 neurons. After 7 days of recirculation (Figure 2D), optical density in the CA1 pyramidal cell layer appeared higher than after 3 days. Optical density in the other layers of CA1 were reduced, and the pyramidal layers of CA3 and CA4 were darker than other gray matter. Staining by cresyl violet (Figure 2H) was more intense than in controls in the CA3 pyramidal cell layer 7 days after carotid occlusion.

Normalized rCw, defined by Equation 1, ranged from 85% at Layer I of the auditory cortex to 121% in the paraventricular nucleus of the hypothalamus in autoradiographs from control brains (Table 1). Only 1 posterior circulation region showed a significant difference from control (p<0.01) between 10 minutes and 7 days after carotid occlusion, as might be expected by chance.

Normalized rCw in all hippocampal regions did not differ significantly from control values at 10 minutes or 1 hour after the 5-minute bilateral carotid occlusion. However, normalized rCw in layers IV and VB of the frontal cortex was reduced and in the internal capsule was elevated at 10 minutes; normalized rCw in Layer I of the frontal cortex, the globus pallidus, the anteroventral nucleus of the thalamus, and Layer I of the auditory cortex was significantly elevated at 1 hour. Normalized rCw in the caudate-putamen and internal capsule at 1 day was also elevated.

One day after carotid occlusion, normalized rCw was elevated by 16% in the CA4 pyramidal layer and by 22% in the dentate gyrus of the hippocampus compared with controls. Elevations of 15% and 17% were observed 3 days after occlusion in the CA3 and CA4 pyramidal cell layers, whereas normalized rCw was reduced by 26% in the CA1 pyramidal cell layer at 3 days. Seven days after occlusion, normalized rCw remained elevated by 15% in the CA3 cell layer but did not differ from control in the CA1 region.

Figure 3 relates brain radioactivity as dpm/mg in unoperated gerbils to time after intravenous injection of 450 μCi/kg [14C]palmitate. In both the hippocampus and the remaining brain, radioactivity increased between 5 and 20 minutes after injection then declined by 4 hours to steady-state values that did not differ significantly from the 24- or 48-hour values. This time course
corresponds to that of brain radioactivity following $[^{14}C]$palmitate administration in rats and indicates that brain radioactivity at 4 hours approximates the steady-state value of $[^{14}C]$palmitate that has been incorporated into brain structures.

Hippocampal sections stained with toluidine blue demonstrated pathologic changes in the CA1 pyramidal cell layer of the hippocampus after bilateral carotid occlusion (Figure 4) corresponding to previously reported delayed neuronal death. The general structure of pyramidal neurons in CA1 was preserved 1 day after recirculation, but by 3 days many neurons had been lost. By 7 days, there was a marked accumulation of phagocytic cells.

**Discussion**

Three to 7 days after a 5-minute bilateral carotid artery occlusion in awake gerbils, hippocampal regions show both significant elevations and reductions in normalized rC, indicating corresponding changes in the rate of regional incorporation of plasma palmitate relative to incorporation into regions of the posterior cerebral circulation. At 3 days, normalized rC is reduced in the CA1 pyramidal cell layer of the hippocampus, whereas at 3-7 days it is elevated in the CA3 and CA4 layers as well as in the dentate gyrus. These changes correspond to irreversible neuronal death of CA1 pyramidal cells and to reversible morphologic changes in the CA1, CA4, and dentate regions.

Insofar as ischemia results in an increase in brain free fatty acids for only about 30 minutes due to activation of lipases and hydrolysis of endogeneous lipids, changes in rC between 1 and 7 days do not reflect an altered brain pool of free palmitate. In gerbils, as in rats, brain radioactivity 4 hours after $[^{14}C]$palmitate injection has reached a steady state value that does not differ from the 24- and 48-hour values. At 4 hours, radioactivity is mainly within phospholipids (primarily phosphatidylethanolamine and phosphatidylinositol).

Bilateral carotid occlusion in gerbils equally and severely reduces blood flow in all regions supplied by

![Autoradiograph Cresyl violet](image_url)

**Figure 2.** Dorsal hippocampus 4 hours after injection of $[^{14}C]$palmitate. Autoradiographs. A: control; B, C, and D: 1 hour, 3 days, and 7 days after 5 minutes of bilateral carotid artery occlusion. Cresyl violet-stained adjacent frozen sections. E: control; F, G, and H: 1 hour, 3 days, and 7 days after occlusion.
the anterior cerebral circulation. The hippocampus is particularly vulnerable, however, responding acutely and chronically with metabolic and morphologic changes that are region-specific. Ischemia as well as excess neuronal activity may contribute to these changes. Although no gerbil convulsed during or following carotid occlusion, excess spontaneous neuronal activity as well as increased rCMR$_{gl}$ have been dem-

Table 1. Normalized Four-Hour Brain Radioactivity Before and After 5 Minutes of Bilateral Carotid Artery Occlusion in Awake Gerbils

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Control</th>
<th>10 min</th>
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<th>3 days</th>
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Each value is mean±SEM (n = 6–8).
PYR, pyramidal cell layer; N, nucleus; VPM, ventral posterior median. Numbers in parentheses refer to reference regions for rat brain.

*Differs significantly from control, p<0.01, by Dunnett's multiple comparison procedure.
Irreversible ischemic cell death in CA1 pyramidal neurons is just evident histologically at 1 day (slight clumping of nuclear chromatin, altered tubulin and creatine kinase BB-isoenzymes), but it is fully evident at 2 days (cytoplasmic clefts, clumping of nuclear chromatin) when spontaneous electrical activity is absent.

CA1 neurons are clearly absent by 3 days after 5-minute ischemia in awake gerbils (Figure 4). Post-ischemic histologic changes in the CA2 and CA3 pyramidal cells of the hippocampus have been characterized as reactive change and ischemic cell change, respectively, and are reversed by 21 days after the ischemic insult. Immediately after a 5-minute carotid occlusion, staining for tubulin in the subiculum-CA1 and CA2 regions of the hippocampus is reduced. At 12–24 hours after a 5-minute bilateral carotid occlusion, the cytoplasm of many CA4 neurons is hyperchromatic and vacuolated, and some CA2 neurons are swollen and contain eccentric nuclei. Reactive change in CA2 cells has been ascribed to increased production of RNA and basic protein, leading to restoration of Nissl bodies.

Reduced normalized rC^ in the CA1 region at 3 days after carotid occlusion corresponds to the phenomenon of delayed ischemic cell death of the pyramidal neurons. The reduction is not caused by opening of the

FIGURE 3. Time course of radioactivity in whole brain and hippocampus following injection of [14C]palmitate (450 μCi/kg i.v.) in awake gerbils. Points are mean ± SEM for 3–7 gerbils.

FIGURE 4. Toluidine blue-stained sections from hippocampus and CA1 pyramidal cell layer in control gerbil (A, ×22; B, ×100) and 3 days (C, ×100) and 7 days (D, ×100) after 5 minutes of bilateral carotid occlusion. Regions corresponding to B–D are marked with a square in A.
blood–brain barrier at 3 days,\(^{3,18}\) which would allow protein-bound \([\text{14C}]\) palmitate into the brain.\(^{3,4,41}\) The dissociated tracer enters normally by diffusing through the cerebral capillary endothelium. Seven days after carotid occlusion, glial activation and phagocytic invasion (Figure 3)\(^{5}\) possibly contribute to the return of normalized \(r_{\text{CMR}}^\text{sk}\) to control levels.

The time course of normalized \(r_{\text{CMR}}^\text{sk}\) in the CA1 layer after bilateral carotid occlusion does not correspond to that of either glucose or protein metabolism. \(r_{\text{CMR}}^\text{sk}\) and spontaneous neuronal activity are increased within 10 minutes after the ischemic insult, whereas \(r_{\text{CMR}}^\text{sk}\) is reduced, and spontaneous activity is absent at 2 days.\(^{5,4}\) In vivo protein synthesis measured with \([\text{14C}]\) leucine is reduced irreversibly at insult and after 2 hours of recirculation.\(^{5}\)

\(r_{\text{CMR}}^\text{sk}\) is unchanged in the CA2 and CA4 regions following 5 minutes of occlusion,\(^{3}\) and \([\text{14C}]\) leucine incorporation into the CA2/CA3 pyramidal cell layer, although elevated at 2 hours, is normal by 4 hours.\(^{3}\) On the other hand, elevated normalized \(r_{\text{CMR}}^\text{sk}\) in the CA3 and CA4 pyramidal cell layers and the dentate gyrus between 1 and 7 days suggests that long-term recovery in these hippocampal regions requires increased synthesis or turnover of palmitate-containing lipids. Increased incorporation of plasma palmitate into brain may compensate for accelerated hydrolysis of endogenous lipids and reduced incorporation of palmitate into brain lipids during the ischemic insult.\(^{34,36,40}\) Recovery also is accompanied by accelerated fatty acid elongation 30 minutes after the insult.\(^{44}\)

In nonhippocampal regions supplied by the anterior cerebral circulation, occasional changes in \(r_{\text{CMR}}^\text{sk}\) are observed at 1 hour and 1 day and, unlike the 10-minute values, probably do not reflect differences in free palmitate concentrations resulting from ischemia (see above). Some of the involved regions have been shown in other studies to be affected by prolonged ischemia in rats and gerbils.\(^{17,19,21}\) Bilateral carotid occlusion has no effect on normalized \(r_{\text{CMR}}^\text{sk}\) in regions supplied by the posterior cerebral circulation.

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