Cortical Activity, Ionic Homeostasis, and Acidosis During Rat Brain Repetitive Ischemia

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Recent data strongly suggest that repetitive ischemic episodes have an adverse cumulative effect on development of edema and tissue damage. We wanted to assess further whether special risks such as exacerbation of extracellular acidification reflecting progressive exhaustion of the capacity to buffer H+ in the extracellular space are associated with repeated short ischemic insults. We monitored spontaneous electrical activity, extracellular direct-current potential, extracellular H+ activity, and tissue Pco2 in the cerebral cortex of rats subjected to four cycles of 3-minute ischemia produced by four-vessel occlusion with 27-minute reperfusion after each insult. Except for electrical activity, which failed to recover fully from the first ischemic insult, all parameters returned to a level close to normal after each reperfusion. Changes during ischemia did not evolve with repetition of the insult. Electrical silence occurred within approximately 20 seconds after the onset of each ischemic episode and always preceded the steep drop of direct-current potential, indicating ischemic depolarization. Each four-vessel occlusion immediately initiated a steep rise of tissue Pco2 and extracellular H+ activity, with extracellular H+ activity reaching a maximum within approximately 145 seconds. Changes in extracellular H+ activity during each recirculation period consistently included an additional and short-lasting increase associated with repolarization, a rapid decrease closely related to that of tissue Pco2, and a slow progressive return to normal. These results suggest that short, repetitive ischemic episodes severe enough to produce cell membrane depolarization and maximum acidosis of the neuronal microenvironment do not have a deleterious cumulative effect on the studied parameters, in particular, on interstitial acidosis. (Stroke 1990;21:1192-1198)

Tomida et al1 recently reported a cumulative effect of repeated ischemic insults in the gerbil. They found that three separate 5-minute bilateral occlusions of the common carotid arteries spaced at 1-hour intervals produced more severe brain edema and tissue injury after 24 hours of recirculation than a single 15-minute ischemic episode. Further work from the same laboratory showed that single and repeated ischemic insults present different patterns of abnormal calcium accumulation.2 These results may have important clinical implications, particularly for neurologic procedures that require temporary clipping of cerebral vessels.3 4

The purpose of this study was to test the hypothesis that detrimental effects of repetitive ischemia may be linked to exacerbation of extracellular acidosis resulting from progressive exhaustion of the brain tissue capacity to buffer H+. We examined the effects of repeated transient ischemic insults on cerebral cortical function, ionic homeostasis, and acid-base balance.

Materials and Methods

We simultaneously monitored spontaneous electrical activity by use of an electroencephalograph (EEG), extracellular direct-current (DC) potential, interstitial H+ activity ([H+]i), and tissue partial pressure of carbon dioxide (Pco2). Four consecutive episodes of cerebral ischemia (3 minutes) and recirculation (27 minutes) were produced by temporary occlusion of both carotid arteries in rats, after previous coagulation of the vertebral arteries. The experiments were concluded by cardiac arrest.

We used 15 adult male Wistar rats (300-350 g, Bantin and Kingman, Grimston, Hull, UK) that had permanent access to food pellets and tap water. The animals were premedicated with atropine sulfate (15-30 μg/kg i.m.), and anesthesia was maintained throughout the experiments with 1-2% halothane in...
O$_2$:N$_2$O (1:1) delivered through a mask or an endotracheal tube. A femoral artery was catheterized for continuous recording of the mean arterial blood pressure and repeated measurements of blood gases and pH, and a femoral vein was cannulated for intravenous administration of drugs. After tracheostomy, the animals were relaxed with tubocurarine (1 mg/kg i.v., repeated every 1–2 hours) and ventilated with a rodent ventilator (Harvard Apparatus Co., Edenbridge, UK) at a rate of 75 cycles/min with appropriate stroke volume to maintain arterial P$_{CO_2}$ within the range of 28–34 mm Hg. If necessary, small amounts of sodium bicarbonate (8.4% wt/vol) were given intravenously to correct for plasma acidosis. Body temperature was kept at 37°C throughout the experiment with a feedback-controlled heating pad.

Common carotid arteries were isolated and separately encircled with a loose-fitting suture, with both ends of the suture passing out of the wound through an approximately 30-mm polyethylene tube (0.8 mm o.d.–0.6 mm i.d.). With these exteriorized snare ligatures, artery occlusion was achieved by gently pulling tight the two ends of the suture; the tautness was maintained during the ischemic period by pinching the catheters with small vascular clamps. Vertebral arteries were exposed through a dorsal cervical incision and bipolarly cauterized under the operating microscope, and the incision was closed.$^5$ 6

A unilateral craniectomy (4-mm diameter), centered halfway between the bregma and lambda sutures, exposed part of the right frontal and parietal cortex. The dura matter was carefully removed with the help of an operating microscope to avoid any damage to the pial blood vessels. A plastic cylinder (10-mm diameter, 5-mm height) was sealed to the skull around the craniotomy and later filled with liquid paraffin.

Tissue P$_{CO_2}$ was monitored from the cortical surface using a miniature P$_{CO_2}$ electrode (ref. MI-720, Microelectrodes, Inc., Londonderry, N.H.) according to Kraig et al.$^7$ Tissue P$_{CO_2}$ electrodes were carefully calibrated at 35°C before each experiment using three gas mixtures containing 5%, 10%, or 20% CO$_2$ and calibration was validated again after the experiment. The 50% and 75% response times of the P$_{CO_2}$ electrode were around 10 and 60 seconds, respectively. Extracellular H$^+$ activity and DC potential were measured with a double-barreled, ion-selective microelectrode$^8$ with its tip inserted 0.5–1 mm into the cortex, as close as possible to the P$_{CO_2}$ sensor. Ion-selective microelectrodes were constructed, filled with Proton-Cocktail (Fluka Chemie AG, Buchs, Switzerland) and calibrated at 35°C as previously described.$^9$ Time constant (63% response time) of H$^+$ measurements was less than 0.5 seconds.

Spontaneous EEG activity was recorded across the cortical region from which P$_{CO_2}$ was measured, using two silver electrodes incorporated into the P$_{CO_2}$ sensor and placed 3 mm apart. The parameters for EEG recording consisted of amplification approximately $20\times10^3$ times, corresponding to a sensitivity of approximately 10 $\mu$V/mm and analog low-pass 1–32-Hz filtering (RM Dynograph Recorder, Beckman Instruments Div., Fullerton, Calif.). Spectral analysis of the amplified and filtered EEG signal and processing of all other physiological variables was carried out on a microcomputer (HP Vectra, Hewlett-Packard Co., Sunnyvale, Calif.) equipped with an analog-to-digital converter (DASH16, Metabyte, Keithley Instruments Ltd., Reading, UK). A dedicated application program written in ASYST (MacMillan Software Co., Keithley) allowed all parameters to be continuously acquired, displayed, and stored. Linear spectra of consecutive EEG data sections (4- or 16-second periods, 128-Hz sampling rate)$^{10,11}$ were computed using the ASYST Fast Fourier Transformation command.

Preliminary experiments showed that components of the EEG linear spectrum greater than 6 Hz were always slower to recover than low-frequency waves. On this basis, the averaged amplitude of the EEG linear spectrum computed over the frequency window (6–21 Hz) for each epoch (4 or 16 seconds) was taken as an index of cortical electrical activity. All other physiological parameters were digitized at a 4-Hz sampling rate and converted on-line to their respective absolute values using precalibrations. In particular, the potentiometric signal issued from the H$^+$ electrode was converted and stored as H$^+$ activities (neq/l). Experiments were selected for further analysis on the condition that EEG became isoelectric shortly after bilateral carotid artery occlusion. Statistical analysis was performed using the Student's paired $t$ test.

After a 30-minute control period, the animals were subjected to four consecutive episodes of cerebral ischemia (3 minutes) and recirculation (27 minutes), followed by cardiac arrest (intravenous injection of 2 ml of 2 mol/l potassium chloride solution). We chose ischemic episodes as short as 3 minutes because preliminary experiments revealed that peak acidosis of the neuronal microenvironment is already reached after approximately 145 seconds of four-vessel occlusion. The 27-minute delay separating two consecutive ischemic episodes was selected because it allows [H$^+$], to return to a level close to normal.

**Results**

All values are mean±SEM. Arterial blood Po$_2$ was always about 120 mm Hg and P$_{CO_2}$ kept in the range of 28–34 mm Hg. Blood glucose in this series of experiments was $10.0±0.9$ mmol/l ($n=12$) and did not change markedly during the experimental procedure. The mean arterial blood pressure showed a typical reactive hypertension during each cerebral ischemia, without any changes relating to the repeated insults (Table 1).

Figure 1 shows the evolution of a representative EEG linear spectrum during the first ischemia/recirculation episode. Figure 2 shows changes in the average amplitude of the same linear spectrum computed in the 6–21-Hz frequency window, together
with those in DC potential (top panel). Spontaneous electrical activity became silent within 15–30 seconds after the onset of ischemia. This event always preceded the disturbance in ionic gradients as indicated by a steep fall in DC potential. Disturbance in ionic gradients subsequent to four-vessel occlusion occurred as early as after cardiac arrest; delays between EEG silence and drop in DC potential during ischemia or subsequent to cardiac arrest were 84±7.7 (n=12) and 83±3.8 seconds (n=14), respectively. The delay between electrical silence (loss of function) and steep fall in DC potential (ischemic depolarization) during the first ischemia was shorter than that from subsequent ischemic insults or after cardiac arrest (Table 2).

On reperfusion, EEG recovery was quite slow and progressive (Figure 1), in particular for the higher frequency components of the spectrum (Figure 2, top panel), which were not back to their control level even after 27 minutes of recirculation. In most cases, EEG never fully recovered to its initial activity. The index for spontaneous EEG activity (averaged amplitude of the linear spectrum in the window of 6–21 Hz) only returned to 71±4.1% (n=14) of control at the end of the first 27-minute reperfusion period. The subsequent episodes of ischemia/reperfusion did not produce any further EEG deterioration (Figure 3). Each time, EEG index recovered to a level of approximately 70% of control, which was not significantly different from that reached at the end of the first reperfusion. Disturbance of ion homeostasis (a steep fall in DC potential) in the subsequent transient ischemia did not last significantly longer or shorter than that produced by the first ischemic insult (Figure 3). Depolarization lasted 158±9 seconds (n=9), whereas 844±58 seconds (n=14) were necessary for the 6–21-Hz EEG waves to return to 50% of control.

The bottom panel of Figure 2 shows the corresponding changes in [H+] and PtcO2. Three-minute four-vessel occlusion immediately initiated a steep rise of [H+] from 54±4 neq/l (pH 7.27; n=12) to a maximum of 220±17 neq/l (pH 6.66; n=12) reached within approximately 145 seconds. Extracellular H+ activity increased linearly from the onset of ischemia until the alkalotic shift of [H+] associated with cell membrane depolarization (Figure 2, bottom panel), at a rate of 102±8.4 neq • l⁻¹ • min⁻¹ (n=11). Brain tissue acidification was associated with a sharp increase in PtcO2, from 76±3.3 to 166±14 mm Hg (n=9). Control PtcO2 values were higher than expected from previous measurements.7 Despite care to avoid such effect, this could have resulted from an excessive pressure of the Pco2 electrode against the pial surface or from a small decrease in cerebral blood flow subsequent to cauterization of the vertebral arteries. In contrast to EEG, at the end of each recirculation interval, [H+] and PtcO2 always returned to a level that was not significantly different from that of the control period (Figures 4 and 5).

Recovery of [H+] during reperfusion consistently included three successive phases (Figure 2, bottom panel, solid line). The first consisted of a short-lasting increase of [H+] associated with brain tissue repolarization, indicating a brief further acidification of the extracellular space. This phenomenon was particularly noticeable when the recovery of ionic gradients was rapid. The second was marked by a rapid decrease of [H+], closely related to that of PtcO2, and the third by a slow and progressive return of [H+] to normal values or to a level slightly higher. On the
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Contrary, $\text{PtCO}_2$ recovery started immediately after the reopening of the carotid arteries and fell quickly to a level lower than that of the control period before slowly returning to its baseline. Three-minute ischemia increased $[\text{H}^+]_e$, to a maximum level not significantly different from that measured 3 minutes after cardiac arrest ($220\pm17$ and $213\pm18$ neq/L, respectively, $n=10$, Figure 4). However, the rate of acidification of the extracellular space after four-vessel occlusion was significantly slower than that after cardiac arrest ($102\pm8.4$ neq·L$^{-1}$·min$^{-1}$, $n=11$ and $158\pm21$ neq·L$^{-1}$·min$^{-1}$, $n=10$, respectively). The $\text{PtCO}_2$ level reached 3 minutes after cardiac arrest ($200\pm16$ mmHg, $n=8$) and was also significantly higher than that after four-vessel occlusion ($166\pm14$ mmHg, $n=9$, Figure 5).

**Table 2. Delay Between Electroencephalographic Silence (Functional Loss) and Cell Membrane Depolarization (Direct-Current Potential)**

<table>
<thead>
<tr>
<th>Ischemic insult (3-min)</th>
<th>Delay (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59±9.5</td>
</tr>
<tr>
<td>2</td>
<td>69±9.5*</td>
</tr>
<tr>
<td>3</td>
<td>94±11†</td>
</tr>
<tr>
<td>4</td>
<td>93±10‡</td>
</tr>
<tr>
<td>Cardiac arrest</td>
<td>81±6‡</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

* $p<0.05$ compared to first ischemia delay.
† $p<0.001$ compared to first ischemia delay.
‡ $p<0.01$ compared to first ischemia delay.

**Discussion**

The global cerebral ischemia produced in rats by four-vessel occlusion presents several advantages: simple preparation, high rate of predictable ischemic neuronal damage, production of a square wave insult that can be repeated, and proper characterization. With this model, local cerebral blood flows were reported to decrease to less than 3% of control values in neocortex, striatum, and hippocampus, which is a level unable to sustain cerebral function and ionic homeostasis. In our study, in animals in which EEG became silent shortly after carotid artery occlusion, there was no significant difference between changes that occurred subsequent to four-vessel occlusion and those after cardiac arrest, except...
for rate of acidification of the extracellular space and maximum Ptco2 reached during ischemia. These findings indicate that very severe global cerebral ischemia was achieved, but was not identical to that resulting from cardiac arrest.

Changes in EEG, DC potential, [H+]e, and Ptco2 observed in these experiments were qualitatively similar to those observed with other rat models in which a single ischemic insult was produced, or with rats subjected to severe anoxia. Three-minute four-vessel occlusion was sufficient to produce a long-lasting and possibly irreversible damage to the function of some neurons, but resulted in only a temporary breakdown of the cellular energy production on which transmembrane ionic gradients depend. Electrical activity was also much slower to recover after each ischemic episode than the other parameters studied.

These results support the theory that the function of the central nervous system is more vulnerable to ischemia than mechanisms maintaining transmembrane ionic gradients and acid-base homeostasis. They also confirm previous work demonstrating that failure of cortical neuronal function is more sensitive to reduced blood flow than cellular ionic homeostasis. Experiments associating EEG monitoring and 31P nuclear magnetic resonance study of cerebral metabolism during histotoxic hypoxia recently suggested that recovery of electrical activity may require both complete restoration of phosphocreatine and inorganic phosphate and normalization of intracellular pH.

The interval between loss of electrical activity and abrupt drop of DC potential represents a transient "penumbral" condition (loss of function with preservation of cellular ionic homeostasis), which was shorter during the first ischemia than during the subsequent ischemic insults or after cardiac arrest (Table 2). Previous results of Siemkowicz and Hansen indicated that this interval is prolonged when preischemic energy stores are higher. Therefore, our data may suggest that the first ischemic insult produced a stress response with subsequent elevation in blood glucose that resulted in a relatively increased brain glucose before the subsequent insults. The fact that blood glucose did not change markedly during the experimental procedure does not support this hypothesis. If one assumes that depolarization occurred at a similar level of energy depletion during each of the repeated ischemic episodes, the duration of DC potential negativation (depolarization) with four consecutive episodes of 3-minute ischemia followed by 27-minute reperfusion. Values of EEG index (averaged amplitude in the window 6–21 Hz expressed in percent of control; lower graph) were computed during the last 4 minutes of each reperfusion period. Note that EEG failed to recover fully after the first ischemic episode, but returned to a similar level (~70%) at the end of each recovery that followed subsequent ischemic insults. Time during which brain cells remained depolarized did not increase with repeated ischemia (upper graph). Ctrl, control period; 1.1–1.4, ischemic episodes 1–4. Values are mean±SEM. **p<0.01, Student's paired t test.

**Figure 3.** Electroencephalographic (EEG) recovery and duration of direct-current (DC) potential negativation (depolarization) with four consecutive episodes of 3-minute ischemia followed by 27-minute reperfusion. Values of EEG index (averaged amplitude in the window 6–21 Hz expressed in percent of control; lower graph) were computed during the last 4 minutes of each reperfusion period. Note that EEG failed to recover fully after the first ischemic episode, but returned to a similar level (~70%) at the end of each recovery that followed subsequent ischemic insults. Time during which brain cells remained depolarized did not increase with repeated ischemia (upper graph). Ctrl, control period; 1.1–1.4, ischemic episodes 1–4. Values are mean±SEM. **p<0.01, Student's paired t test.

**Figure 4.** Evolution of extracellular H+ ([H+]e) during four consecutive episodes of 3-minute ischemia followed by 27-minute reperfusion. Cardiac arrest (KCl, intravenous injection) concluded the experiment. After 27-minute reperfusion, [H+]e activity was always slightly higher than that of control period but did not show a cumulative effect (lower graph). Peak [H+]e levels during repetitive ischemia remained within the same range (upper graph), not significantly different from those reached 3 minutes after cardiac arrest (single filled triangle). Ctrl, control period; 1.1–1.4, ischemic episodes 1–4. [H+]e is given as activity in neq/L (mean±SEM). **p<0.001; *p<0.05, Student's paired t test.
insults, one can propose that the increase in time between onset of ischemia and ischemic depolarization reflects a decrease in brain tissue energy demand.

Except for EEG, which did not fully recover after the initial insult (Figure 1), all parameters returned to a level close to normal after each reperfusion, and the changes during each ischemic episode were remarkably similar (Figures 2–5). These results suggest that repeated 3-minute ischemic episodes severe enough to produce cell membrane depolarization and peak acidosis do not have an adverse cumulative effect on the parameters under study. Our data relating to ionic homeostasis (DC potential) confirm preliminary results of Urbanics et al,22 who observed that, in gerbils, extracellular K+ recovers rapidly and almost completely after each of three 5-minute ischemic episodes. However, their results relating to brain extracellular pH contrast with ours in that only partial recovery of extracellular pH was achieved after reopening of the carotid arteries, and cumulative acidosis occurred with repeated insults.

Klatzo and his associates1,2 have collected clear evidence of a deleterious cumulative effect of repetitive ischemia in gerbils, which may result from progressive impairment of the microcirculation.23 We found no sign of adverse cumulative effect of repetitive ischemia in our experiments, but differences in species, experimental procedure, and in variables studied preclude closer comparison of our data with theirs.

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References


**KEY WORDS** • cerebral ischemia • electroencephalography • acidosis • rats
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