Pathophysiology of Ischemic Cell Death: III. Role of Extracellular Factors

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SUMMARY The purpose of this study was to determine the effect on cell survival of extracellular changes that occur during ischemia, over and above the depletion of O\textsubscript{2} and substrate. Rabbit retinas were deprived in vitro of both O\textsubscript{2} and substrate, and then returned to control medium for 4 h before recovery was assessed by measuring protein synthesis, glucose utilization, and tissue water. Experimental conditions were altered in various ways during the period of O\textsubscript{2} and substrate deprivation in order to modify the changes taking place in the extracellular fluid as a result of the failure of energy metabolism. When O\textsubscript{2}-free, substrate-free extracellular electrolyte solution was added to the retinas to reduce the ischemia-induced changes in the extracellular fluid, there was marked reduction in irreversible damage. But when energy-deprived retinas were exposed to retinas that had already been ischemic, or to interstitial fluid from ischemic retinas, there was an increase in irreversible damage. Removing Ca\textsuperscript{2+} from the extracellular fluid during the period of energy deprivation increased the damage due to short deprivations in a restricted volume of extracellular fluid, but reduced the damage from longer deprivations in a large volume of extracellular fluid. The results demonstrate that several changes occur in the extracellular fluid during ischemia that significantly affect recovery.

THE CONSTANCY OF THE COMPOSITION OF the interstitial fluid (ISF) depends on the circulation of the blood. Interruption of circulation leads to a myriad of changes, with the rate of change of each constituent depending on its net flux across the plasma membranes of the cells. Since the solutes involved in energy metabolism normally exhibit the largest fluxes, they are the first to show appreciable change. However, other constituents are affected when steady state conditions are altered by the failure in energy metabolism. Electrolytes, whose distributions across plasma membranes are normally closely regulated, may show large changes (e.g., the rise in extracellular K\textsuperscript{+} and fall in Ca\textsuperscript{2+}); and catabolic products that accumulate in the cells may also appear in the extracellular fluid (e.g., lactic acid and NH\textsubscript{3}\textsuperscript{+}).

The purpose of this study was to assess the effects on the cells’ survival of the extracellular changes that occur during ischemia, over and above the depletion of O\textsubscript{2} and substrate. Three types of experiments were performed. In the first, the changes in extracellular fluid (ECF) composition that occur during ischemia were reduced by dilution with electrolyte solution containing neither O\textsubscript{2} nor substrate. In the second, exposure to the ischemia-induced changes in the ECF was increased by putting the test retinas in contact with ECF from conditioning retinas that had already been ischemic for 20 or 40 min. In the third type of experiment, the role of Ca\textsuperscript{2+} was examined by markedly reducing the Ca\textsuperscript{2+} concentration in the ECF of the energy-deprived cells.

**Methods**

The procedures for isolating and incubating the retinas and for measuring rates of glucose utilization and protein synthesis have been previously described\textsuperscript{1} and will be reviewed here only briefly. Retinas weighing about 75 mg (wet wt) were removed from New Zealand White rabbits and maintained at 37 ± 0.1°C in a medium that resembled human CSF with respect to electrolyte composition and 38 organic constituents. The medium was equilibrated with a gas mixture of 40% O\textsubscript{2}, 5% CO\textsubscript{2}, and 55% N\textsubscript{2}. (See \textsuperscript{4} for detailed
description of composition and preparation of the medium, and for a photograph of the incubation boat).

Control retinas remained in this medium for the duration of the incubation, i.e. for up to 5 h. Test retinas were subjected to different types of ischemic insult, as described below, and then returned to the control medium for 4 h before their recovery was evaluated by measuring water content, 2-deoxyglucose (2-DG) uptake, and leucine incorporation into protein. For the latter measurements, $^{14}$C-2-DG and $^3$H-leucine were added together to the medium for a $\frac{1}{2}$ h period ending 15 min before harvesting, the 15 min "chase" having been shown sufficient to clear the tissue of unphosphorylated 2-DG and unincorporated leucine.$^1$ At the time of harvest, the optic nerve stump was discarded; the retina was touched to glass to remove excess surface wetting; and the retina was weighed wet and again after lyophilization to obtain dry weight and total water. The retina was solubilized with Nuclear-Chicago Solubilizer (NCS), and differentially counted for $^{14}$C and $^3$H in a liquid scintillation counter with samples of the labeling medium that had been treated similarly.

**Different Types of Ischemic Insult**

In all experiments, the retinas were first removed from the control medium and immersed for 1 min in a large volume of solution that contained normal electrolytes but that was devoid of all organic solutes and was equilibrated with 95% N$_2$ and 5% CO$_2$. Depending on the type of experiment, the solution bathing the cells was then altered with respect to volume, previous contact with ischemic cells, or Ca$^{2+}$ content; but in all experiments the retinas remained completely deprived of O$_2$ and exogenous substrate for the duration of the ischemic insult. After the period of ischemia, they were always returned to the control medium for 4 h before being tested for irreversible damage.

To examine the effects of a minimum volume of ECF during energy deprivation, retinas were transferred in the nitrogen atmosphere to a small Teflon container that was tightly sealed with a silicone lid (see 1 for photograph) so that they had only their own interstitial fluid (about 35 $\mu l$) as extracellular fluid during the deprivation (ECF $= 1 \times$ ISF). In other experiments, the Teflon container contained 70 $\mu l$, 100 $\mu l$, or 500 $\mu l$ (using a larger container) of the O$_2$-free, substrate-free electrolyte solution; so that the total ECF present during the energy deprivation was approximately $3 \times$, $4 \times$, or $15 \times$ ISF respectively. In still other experiments, the retina was left motionless in 20 ml of the electrolyte solution in an incubation boat (see 2 for photograph). Since exchange under these circumstances was limited more by diffusion than by volume, we have characterized the condition as ECF $= \infty$. In some experiments, the boat was rocked to cause continuous motion of the retina through the solution and to further increase the effectiveness of the exchange.

Two types of experiments were performed to examine the effects of increasing the retina's exposure to ischemic ISF. Both were performed in the larger-sized Teflon container. In one, a test retina was interposed for 20 min in a sandwich configuration, between two retinas that had already been in the container for 20 min. The control retina for these experiments was similarly interposed between two retinas that had not previously been ischemic. In the other, two conditioning retinas were energy-deprived for 40 min in the Teflon container with 100 $\mu l$ of electrolyte solution. They were then discarded; the volume of the electrolyte solution remaining was determined without exposure to O$_2$; and the test retina was immersed in it for a 30 min period of energy deprivation. A control retina was subjected to a 30 min deprivation in the same volume of fresh electrolyte solution.

The effect of reducing ECF Ca$^{2+}$ during the period of energy deprivation was tested in experiments in which retinas were energy-deprived in the Teflon container with 100 $\mu l$ of Ca$^{2+}$-free electrolyte solution, or while motionless in the incubation boat containing 20 ml of Ca$^{2+}$-free solution. The test solution was prepared by replacing the CaCl$_2$ in the control electrolyte solution, isosmotically, with NaCl.

**Results**

**Effects of Dilution of the Interstitial Fluid During Energy Deprivation**

When progressively more O$_2$-free, substrate-free electrolyte solution was added to energy-deprived retinas, to increase the volume of their ECF from the volume of the interstitial fluid to a volume 15-fold greater, there was a marked improvement in recovery of protein synthesis (fig. 1) and water content (fig. 2). The improvement in 2-DG uptake (fig. 3) was less marked but also significant ($p < 0.01$ for 30 min and $p < 0.05$ for 40 min of deprivation). Similarly, there was a marked improvement in the three criteria of recovery when the proportion of the time spent in a large volume of ECF, vs. that spent in a restricted volume, was increased from 0% to 50% or 66% (fig. 4). In the latter experiments, recovery was not affected by whether the period in a restricted volume of ECF occurred during the first half or second half; or during the first, middle, or last third of the deprivation period.

However, when the volume of ECF bathing the deprived retinas was further increased from 15 $\times$ ISF to a volume that was very large relative to diffusion distances, and then effectively increased even more by moving the retina through the large volume, there was an adverse effect on the retina's recovery as indicated by the latter portions of the curves in figures 1–3. An adverse effect was also observed when the time spent in a large volume of ECF was increased above 66% (fig. 4). The uptake of 2-DG appears to have been more sensitive to this effect than protein synthesis or total water, as indicated by a comparison of the curves in figure 4 and by comparing the 40 min curves in figure 3 with those in figures 1 and 2.

**Effect of Exposure to Ischemic Interstitial Fluid During Energy Deprivation**

Two types of experiments were performed to examine more directly the effects of ischemic interstitial
Retinas were deprived completely of O₂ and substrate for 30, 40, or 60 min and then returned to control medium for 4 h before recovery was assessed by measuring leucine incorporation into protein. The effective volume of extracellular fluid present during the deprivation was increased in 6 steps as shown on the abscissa. Some retinas were sealed under 95% N₂-5% CO₂ in a Teflon container without added fluid so that they had only their interstitial fluid (about 35 μl) as extracellular fluid during the deprivation (ECF = ISF). Some were sealed in the container with 70, 100, or 500 μl of O₂-free, substrate-free electrolyte solution so that their ECF was equal to 3x, 4x, or 15x ISF respectively. Some were left motionless in a volume of electrolyte solution (20 ml) that was very large relative to diffusion distances (ECF = ∞); and some were moved through the large volume to increase further the effectiveness of exchange (ECF = ∞ + motion). Values are means ± S.E.M. Number of retinas in parentheses.

Experiment, test retinas were deprived of O₂ and substrate for 30 min while immersed in electrolyte solution that had been in contact with ischemic retinas. The paired controls were similarly deprived while in the same volume of fresh electrolyte solution. As shown in figure 5, recovery of the test retinas was significantly impaired as assessed by all 3 criteria.

Role of Ca⁺⁺

Since an influx of Ca⁺⁺ into the energy-deprived cells may have contributed to the findings described above, a series of experiments was performed to examine the effect of removing Ca⁺⁺ from the ECF during O₂ and substrate deprivation. When the deprivation was for only 30 min and when the cells had access to a relatively small volume of extracellular fluid (ECF = 4x ISF), omitting Ca⁺⁺ caused a reduction in the recovery of leucine incorporation (from 16.2 ± 0.6 (SEM) to 12.8 ± 0.8 nmole/g/min; p < 0.05) and an increase in tissue water (from 6.23 ± 0.19 to 6.99 ± 0.17 ml/g; p = 0.06). However, when the retinas were deprived for 40 min with access to a large volume of extracellular fluid (ECF = ∞), omitting Ca⁺⁺ improved the recovery of both leucine incorporation (from 17.4 ± 0.2 to 20.2 ± 0.4 nmole/g/min, p < 0.005) and 2-DG uptake (from 0.128 ± 0.09 to 0.175 ± 0.02 nmole/g/min; p < 0.01). The pattern of the responses to the 4 combinations of duration and ECF volume is shown in table 2. The results suggest that marginally deprived cells benefit from the Ca⁺⁺ that is...
present in a small volume of ECF (i.e., their recovery was less complete when it was omitted); but that severely energy-deprived cells are further damaged by the Ca\(^{++}\) available in a large volume of ECF (i.e., they were protected by its omission). The recovery of protein synthesis reflected both actions of Ca\(^{++}\), whereas glucose utilization appeared to be more responsive to its potential for damage, and the maintenance of normal water content appeared more responsive to its protective role. A possible explanation for the diphasic action of Ca\(^{++}\) is presented below.

**Discussion**

**Adverse Effects of Ischemic Interstitial Fluid on Survival**

The initial portions of the curves in figures 1–4 demonstrate a protective effect from the dilution of the ischemic interstitial fluid, and the results in figure 5 and table 1 demonstrate an adverse effect from increasing the retina’s exposure to this fluid. The retina’s response in both types of experiment indicates that the development of irreversible damage during an ischemic insult may depend on factors in addition to energy deprivation per se, and the effects of these extracellular factors may be large. For example, retinas that were energy-deprived for 30 min recovered less than 50% of their protein synthetic capacity if the interstitial fluid was undiluted; but they recovered fully if their interstitial fluid had been diluted 15-fold with O\(_2\)-free, substrate-free electrolyte solution (fig. 1). Retinas that were energy-deprived for 30 min while bathed in ECF that had been in contact with ischemic retinas exhibited a 70% greater reduction in their protein synthetic capacity than retinas that were energy-deprived in the same volume of unconditioned electrolyte solution (fig. 5). The toxicity of the ischemic interstitial fluid may have resulted from the addition of substances that were damaging to the cells or from the depletion of beneficial constituents. Both types of change probably occurred.

Substances whose accumulation may contribute to loss of viability during ischemia include K\(^+\), lactate, NH\(_4\)^+, and the products of phospholipid breakdown. The last 2 are of particular interest. Ischemia causes brain NH\(_4\)^+ to increase rapidly to levels that are toxic to the cells. There is also a rapid breakdown of phospholipids, with the production of 4 potentially...
extracellular Ca$^{++}$ might be expected to add to the damaging effects of energy deprivation. In the present experiments, increasing the ECF volume by 4-fold during 30 min of energy deprivation reduced the irreversible damage (assessed by protein synthesis) by 57% if the added electrolyte solution contained Ca$^{++}$, but it reduced the damage by only 28% if the added fluid was Ca$^{++}$-free ($p < 0.05$ for the effect of Ca$^{++}$). Thus, the additional Ca$^{++}$ appears to have accounted for some, but not all, of the protective effect of the added fluid. No experiments were performed to examine the role of Mg$^{++}$.

Implications of the Positive Feedback Feature

If energy deprivation, which may itself be irreversible, leads to a change in the composition of the interstitial fluid that also restricts recovery, it seems apparent that the system has the potential for a positive feedback of serious consequence. This would have two implications with respect to the tissue's response. (1) As the duration of the ischemia exceeds the time for the appearance of irreversible damage, the amount of damage would be expected to increase very rapidly. (2) As the more vulnerable cells are damaged, their effect on the interstitial fluid would be expected to involve neighboring cells, so that there would be a generalized loss of viability. Experimental evidence appears to support both predictions.

In experiments in which the duration of energy deprivation was progressively increased, from durations compatible with full recovery to durations that resulted in a major degree of irreversible damage, one of the features that most distinguished the retinas deprived in a small volume of extracellular fluid from those deprived in a large volume was the high rate at which viability was lost when the tolerable limit was exceeded (see, for example, fig. 2 in 22). When histological examination was performed on retinas subjected to progressively longer periods of energy deprivation, the retinas deprived in a small volume of extracellular fluid showed not only an earlier but also a more generalized and more nearly simultaneous involvement of all cell types; whereas, in retinas deprived in a large volume, some cell types appeared much more resistant than others.

Occlusion of the middle cerebral artery of monkeys may or may not result in infarction, depending on the collaterals; 24 but when an infarction occurs it is usually

### Table 1 Effect of Contact with Ischemic Tissue During Period of Deprivation

<table>
<thead>
<tr>
<th></th>
<th>3$^H$-leucine incorporation</th>
<th>2-DG uptake</th>
<th>Total water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test retinas</td>
<td>17.8 ± 1.5 (5)</td>
<td>0.187 ± 0.010 (5)</td>
<td>6.01 ± 0.13 (5)</td>
</tr>
<tr>
<td>Control retinas</td>
<td>20.4 ± 1.1 (5)</td>
<td>0.215 ± 0.012 (5)</td>
<td>6.07 ± 0.15 (5)</td>
</tr>
<tr>
<td>Test/Control, by paired variate analysis</td>
<td>0.874 ± 0.058 (5)</td>
<td>0.888 ± 0.075 (5)</td>
<td>0.99 ± 0.03 (5)</td>
</tr>
</tbody>
</table>

Retinas were deprived of O$_2$ and substrate for 20 min and then returned to control medium for 4 h before their recovery was assessed. Both test and control retinas were interposed between 2 other retinas during the period of deprivation, but the retinas in contact with the test retina had already been ischemic for 20 min while those in contact with the control retina had not. $3^H$-leucine incorporation and 2-DG uptake are expressed as nmole per g dry wt per min; total water as ml per g dry wt. Values are means ± S.E.M.; number of experiments in parentheses.
that the recovery of brain slices was markedly impaired both because synthesis is impaired and because loss to depletion of endogenous substrates and cofactors, this biphasic response to increasing the volume of ECF is clearly evident in the inflections of the curves in figures 1—4. It indicates that, whereas moderate dilution of the ischemic ISF was beneficial, further dilution had a net adverse effect. And, similarly, as the proportion of the time during which the cells were bathed by a large vs. a small volume was increased above 66%, there was also an increase in the amount of damage, at least with respect to the recovery of 2-DG uptake (p < 0.05).

The adverse effect of a large extracellular volume might be the consequence of the elution of the cells of substances required for recovery. Energy-deprived cells would be expected to be particularly susceptible to depletion of endogenous substrates and cofactors, both because synthesis is impaired and because loss from the cell may be increased owing to increased permeability of plasma membranes and failure of active transport systems. Phizackerley and Fixter found that the recovery of brain slices was markedly impaired when the volume of ECF was increased during O₂ deprivation, and they obtained evidence that the adverse effect of the larger volume was due in part to elution of glutamine. Glutamine depletion of CNS cells has also been observed in vivo during, and following, severe hypoglycemia. Glucose may represent a special case in which there is elution of substrate being transferred between cells. The retinal glia, or Mueller cells, contain glycogen and glucose-6-phosphatase; and they presumably provide glucose to adjacent neurons at times of increased demand or decreased supply. Increasing diffusional exchange between the retina and the electrolyte solution may have deprived the neurons of some of this glucose. It is of interest in this regard that, of the 3 criteria of recovery, the recovery of glucose utilization was the one most impaired by a large increase in the cells' exchange with the ECF.

Biphasic Effect of Increasing ECF Volume During Energy Deprivation

As the volume of electrolyte solution bathing the cells during the period of energy deprivation was increased to more than 15× the volume of interstitial fluid and as exchange was further facilitated by moving the tissue through the larger volume of fluid, there was an increase in damage rather than further improvement. And, similarly, as the proportion of the time during which the cells were bathed by a large vs. a small volume was increased above 66%, there was also an increase in the amount of damage, at least with respect to the recovery of 2-DG uptake (p < 0.05). This biphasic response to increasing the volume of ECF is clearly evident in the inflections of the curves in figures 1–4. It indicates that, whereas moderate dilution of the ischemic ISF was beneficial, further dilution had a net adverse effect.

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An alternative, or additional, explanation for adverse effects of increasing diffusional exchange with the electrolyte solution is the additional Ca++ that is made available. There is considerable evidence that the net flux of Ca++ into ischemic cells contributes

![Figure 6](https://example.com/figure6.png)
importantly to cell death, and, when the cells were deprived of O₂ and substrate for 40 min in a large volume of electrolyte solution, omission of Ca ++ provided significant protection (table 2).

As summarized in table 2, the omission of Ca ++ from the extracellular fluid sometimes impaired recovery and sometimes improved recovery, depending on the circumstances. Both effects were statistically significant. They are explicable if the Ca ++ influx that occurs during energy deprivation had adverse effects both because of the extracellular depletion and the intracellular accumulation. The former predominates when the extracellular fluid volume is restricted and the ischemia is of marginal duration with respect to irreversibility. The latter predominates when extracellular fluid volume is large and energy deprivation prolonged. If this interpretation is correct, altering extracellular Ca ++ concentration will have less potential as a protective measure than reducing Ca ++ permeability, perhaps with Ca ++ entry blockers.

The experiments described here indicate that damage from ischemia is determined not only by the extent of O₂ and substrate deprivation but also by other abnormalities in the cells’ exchange with their surrounding ECF. When extracellular volume was restricted, as in complete circulatory arrest, recovery appears to have been limited by the accumulation of toxic catabolic products, and by the fall in ECF Ca ++. When the energy-deprived cells had access to a large extracellular volume, recovery appears to have been limited by the elution of endogenous substrates or cofactors, and by the availability of excessive amounts of Ca ++. The experiments in which energy-deprived retinas were exposed to a large extracellular volume did not duplicate a condition that occurs in vivo, since the ECF was completely devoid of O₂ and exogenous substrates. However, the retina’s response to these conditions may be relevant to the response of in vivo brain when blood flow is allowed to continue, but at a level that is insufficient to maintain viability. Under these circumstances, recovery has sometimes been less complete than with no blood flow at all.

We still know relatively little about the ECF changes that occur during ischemia, and how they affect recovery. More information will be required before we can fully understand the cells’ response to ischemia and identify the causal sequences that determine irreversibility. More information about the ECF changes in different clinical situations will probably help explain the diversity of the pathological responses, since it is likely that the factors that limit survival will be found to differ, depending on the nature of the insult.

References
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ANGIOGRAPHIC ARTERIAL LUMINAL NARROWING, a common complication of aneurysmal subarachnoid hemorrhage, most frequently has been attributed to vasospasm, implying that an abnormal muscular contraction has taken place in the cerebral vessel.\(^1\-^3\) Although vessel constriction has been observed immediately after hemorrhage, a second phase apparently begins after a few hours and persists for days or weeks. The delayed or persistent phase seems to be associated with the high morbidity.\(^4\-^7\)

Little emphasis has been given to structural alterations in the cerebral arteries, perhaps because the time course over which these changes have been observed has not appeared consistent with the radiographic appearance of constriction. Pathological alterations are generally believed to appear weeks following aneurysm rupture and the magnitude of damage has not been regarded sufficient nor its appearance early enough to explain the reduced luminal diameter seen angiographically.\(^5\-^8\) Recently, morphological alterations, similar to those observed after subarachnoid hemorrhage in man were produced by arterial rupture in the primate.\(^9\-^10\) Within 3 days of subarachnoid hemorrhage (SAH), changes in the intimal and medial layer were apparent and constriction in the vessel, due to loss of compliance, was seen several days before morphological changes became florid, in the second week following subarachnoid hemorrhage.\(^9\-^10\)

Since many patients linger in a vegetative state as a result of cerebral infarction, there has been little opportunity to examine the vessels early in the course of subarachnoid hemorrhage and to correlate these with premortem angiography. In Conway’s series of 12 patients, in only one instance had angiography been carried out and pathological examination conducted within one week of hemorrhage.\(^5\) Premortem arterial narrowing was observed. Mizukami’s series also includes one examination eight days after hemorrhage in which angiography demonstrated constriction and early morphological findings were present.\(^11\) In Crompton’s series, 37% of all patients with cerebral infarction showed arterial constriction on the angiogram but neither temporal nor anatomical correlations were made.\(^4\) Since most histological studies have not concentrated on these earlier subtle changes, we have reviewed our own recent series with the objective of making anatomical-angiographic correlates. Denoting factors bearing upon the development of the angiopathy was a further goal of this project.

Materials and Methods

The seventy-seven patients with fatal subarachnoid hemorrhage (SAH) studied at University of Mississippi Medical Center over the past 15 years were reviewed in a retrospective post-mortem study; both angiograms and tissue slides were available in 38 (50%). In others, tissues were available but angiographic data was lacking. Of the 38 patients with SAH having both angiography and tissue slides, 28 of them had evidence of the rupture of a cerebral aneurysm. While all 38 cases were analyzed as a group, the 28 due to aneurysmal rupture were viewed separately since they repre-
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