Pathophysiology of Ischemic Cell Death:
II. Changes in Plasma Membrane Permeability and Cell Volume

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SUMMARY Isolated rabbit retinas were subjected for various durations to several types of ischemic insult, and then returned to control medium for periods of up to 4½ h before measurements were made of total water, inulin-free water, and plasma membrane permeability as assessed by mannitol penetration into the inulin-free water. Neither anoxia nor substrate deprivation alone, for as long as 50 min, caused significant irreversible swelling, but they were synergistic in combination. Restricting the volume of extracellular fluid during the combined deprivation caused the changes responsible for swelling to occur much sooner. There was a progressive increase in membrane permeability, with a delayed increase in intracellular water beginning about 2 h after the ischemic insult. Cell swelling correlated closely with loss of viability as evidenced by failure to reinstitute protein synthesis, but the swelling appeared to be the consequence rather than the cause of the initial irreversible damage.

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BRAIN SWELLING is a regular consequence of ischemia, and one to be expected on the basis of our understanding of the factors governing the distribution of water. Maintenance of normal intracellular volume depends on the active transport of ions and on the relative impermeability of the plasma membrane, both of which are jeopardized by ischemia; and, since the endothelial cells are subject to the same problems as the parenchymal cells, there may be disruption of the blood-brain barrier with an increase in interstitial fluid as well as intracellular fluid. There is still considerable uncertainty about how much of the extra water is intracellular and how much is extracellular, and particularly about the time course of the changes. It has not been clear, for example, how much swelling occurs before loss of viability of the cells involved. This is a question of some importance since swelling may itself be severely damaging to the cell and could represent a critical link in the causal chain leading to cell death. It has been a difficult problem to study in vivo because the blood brain barrier prevents the introduction of extracellular markers (e.g. inulin), and because the bony calvarium causes even a small amount of swelling to result in an increase in pressure with a reduction in circulation and a host of complicating changes. Though an in vitro preparation precludes study of those components of the ischemic insult were most responsible for cell swelling; to determine the time course of cell swelling and its relation to changes in membrane permeability; and to examine the correlation between cell swelling and irreversible damage.

Methods

The procedures for isolating the retina, for maintaining it under control conditions, and for subjecting it to the various types of ischemic insult, were the same as in the preceding study and will be reviewed here only briefly. Retinas weighing about 75 mg (wet weight) were removed from New Zealand White rabbits and maintained at 37 ± 0.1°C in a control medium that resembled human CSF with respect to electrolyte composition and 38 organic constituents, and that was equilibrated with a gas mixture of 40% O₂, 5% CO₂, and 55% N₂. (See 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 for periods of up to 50 min to one of four types of ischemic insult: (1) complete deprivation of organic substrates, with O₂ present at the control level; (2) complete deprivation of O₂, with substrates present at control levels; (3) deprivation of both O₂ and substrate, with the retina maintained in 20 ml of electrolyte solution equilibrated with 95% N₂ and 5% CO₂; (4) deprivation of both O₂ and substrate with a restricted volume of extracellular fluid. For this last type of experiment, the retina was first immersed in electrolyte solution to remove organic solutes from the extracellular phase and then transferred with only its interstitial fluid, in a nitrogen atmosphere, to a small Teflon container that was tightly sealed with a silicone lid (see for photograph). Following the ischemic insult, the retinas were some-
times removed immediately for analysis, but more often they were returned to the control medium for periods of up to 4¼ h, before their condition was assessed.

To measure the volume of distribution of inulin, or the "inulin space", 14C-inulin was added to the medium 15 min before harvesting, at a specific activity of about 0.8 μCi/ml. 3H-mannitol (specific activity about 4 μCi/ml) was sometimes added to the medium with the inulin to permit a simultaneous determination of the mannitol space. (Previous studies have shown that the inulin labeling of the retina’s extracellular space is about 90% complete in 2 min and that the mannitol labeling is about 90% complete in 0.5 min.) In some experiments, 3H-leucine was added to the medium to measure protein synthesis.

At the time of harvest, the tissue was picked up with forceps applied to the optic nerve stump, and touched quickly to glass to remove excess surface wetting. The nerve stump was cut off and discarded, and the retina was quickly to glass to remove excess surface wetting. The retina proper was placed in a weighing bottle. It was weighed wet and again after lyophilizing, to obtain dry weight and total water. To determine the tissue content of the labeled compounds, the retinas were solubilized with Nuclear-Chicago Solubilizer (NCS) and differentially counted for 14C and 3H in a liquid scintillation counter, together with samples of labeling medium that had been treated similarly (ibid.).

Results

Effects of Different Types of Ischemic Insult on Total Water Assessed Following an Extended Recovery Period

Figure 1 shows the results of a series of experiments in which retinas were exposed for progressively longer periods to four different types of ischemic insult. The retinas were then returned to the control medium for a 4¼ h recovery period before their water content was determined. Deprivation of either O2 or substrate alone had relatively little effect, and there was no evident progression as the duration of the deprivations was increased up to 50 min. Deprivation of O2 and substrate together caused a progressive increase in water as the duration of the deprivation was extended from 20 to 50 min. The increase after 30 min was significant (p < 0.01 for test vs. controls), and there was a dramatic further increase when the duration of the combined deprivation was extended from 40 to 50 min.

The condition having the greatest effect on tissue water was the deprivation of both O2 and substrate with the volume of extracellular fluid (ECF) restricted to the volume of the interstitial fluid, as would occur during circulatory arrest in vivo (see uppermost curve in fig. 1). Retinas subjected to this condition for 20 min still maintained a normal water content, but after 30 min of the ischemic insult there was a marked increase in total water which reached a maximum after 40 min of exposure.

Correlation Between Increase in Tissue Water and Irreversible Damage

Measurements of tissue water were compared with measurements of protein synthesis in retinas that had been subjected to O2 and substrate deprivation, in either a large or small volume of ECF, and then allowed 4¼ h of recovery in control medium (fig. 2). The reduction in the capacity to synthesize new protein provides a measure of the extent of the irreversible damage that has been found to correlate well with other criteria for loss of viability. Comparison between the left and right sides of figure 2 shows a distinct difference between the two types of ischemic insult; but the mirror-image relationship between the solid line and the dotted line on each side reveals a close correlation between the increase in total water and the extent of the irreversible damage as assessed by the failure of protein synthesis.
Time Course of Changes in Cell Water

When retinas were deprived of O₂ and substrate for 40 min in a large volume of ECF, the cells became swollen during the period of deprivation, as indicated by the increases in total water and inulin-free water shown at 0 recovery time on the left of figure 3. Upon return to control medium, there was a prompt reduction in total water (not statistically significant) and in inulin-free water (p < .01). The significance of the latter measurement with respect to the volume of intracellular water is uncertain, since the reduction in inulin-free water was accompanied by some increase in the inulin space and may have reflected inulin penetration into damaged cells (see below) rather than a reduction in cell size. It seems probable that both changes occurred, and that a portion of the swollen cells recovered their normal size whereas others that had been irreversibly damaged became increasingly permeable to inulin.

In contrast to the above, retinas that had been deprived of O₂ and substrate for 30 min in a restricted volume of ECF showed a slight reduction in total water 1½ h after their return to control medium (not statistically significant), followed by a progressive and marked increase in water (p < .001) that continued until the end of the 4½ h recovery period (fig. 3, upper right). These retinas also showed a reduction in inulin-free water followed by a marked increase (p < .001) that was similar to, but somewhat less than, the increase in total water (fig. 3, lower right). The initial reduction in inulin-free water was significant in the retinas examined after ½ h (p < .05) and after 1½ h (p < .005) of recovery. It presumably reflects a transient reduction in cell size, for which other evidence is also available (see Discussion).

Time Course of Changes in Membrane Permeability

Both mannitol and inulin are effective extracellular labels in retina, 6 and, though mannitol (MW 182) is much smaller than inulin (MW about 5,300), the volume of distribution of mannitol is only 9% greater than that of inulin in normal retina (cf. control values in fig. 4). An increase in plasma membrane permeability would be expected to permit penetration of mannitol into the cells before inulin, and this would be revealed as an increase in the difference between the mannitol space and the inulin space. In order to measure the changes in membrane permeability in retinas recovering from the 2 types of ischemic insult, 3H-mannitol and 14C-inulin were added together to the medium 15 min before the retinas were harvested.

When retinas that had been subjected to 40 min of O₂ and substrate deprivation in a large volume of ECF were tested 15 min after their return to control medium, there was a moderate increase in plasma membrane permeability as indicated by an increase in the difference between the mannitol and inulin spaces from the control level of 0.32 ± .02 S.E.M. ml/g to 0.54 ± .05 ml/g. When tested after an additional 4½ h of recovery, the difference between the mannitol and

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**Figure 3.** Retinas were deprived of O₂ and substrate for 40 min while immersed in a large volume of electrolyte solution (left) or for 30 min while sealed in a Teflon container with only their interstitial fluid (right). Measurements of total water and inulin-free water were made at the end of the period of deprivation and following varying periods of recovery in control medium. Measurements on retinas that had not been subjected to an ischemic insult are shown as controls. Results are expressed as ml per g dry wt. Bars show S.E.M.; number of experiments in parentheses.

**Figure 4.** Retinas were deprived of O₂ and substrate for 30 min with the ECF restricted to the interstitial fluid. They were then returned to control medium for periods of from ½ h to 4½ h, and plasma membrane permeability was assessed by measuring mannitol penetration into the inulin-free water. 3H-mannitol and 14C-inulin were added together to the medium 15 min before harvesting, and the difference between the mannitol space and the inulin space was determined as ml per g dry wt (solid line). The changes in total water are shown for comparison (dashed line). Comparable measurements were made on control retinas that had not been subjected to an ischemic insult. Bars show S.E.M.; number of retinas in parentheses.
inulin spaces had returned to normal (0.27 ml/g, 1 retina). The initial increase in the permeability was measured when there was a 16% increase in inulin-free water (fig. 3), and it is consistent with the permeability increases that have been observed with comparable increases in intracellular water following a reduction in medium osmolality. It seems likely therefore that it was a consequence of the swelling rather than a direct effect of the ischemic insult.

The changes in plasma membrane permeability following 30 min of energy deprivation in a restricted volume of ECF are shown in figure 4. The difference between the volumes of distribution of the 2 extracellular markers was increased slightly above the control level ($p < 0.05$) when the retinas were examined $\frac{1}{2}$ h after resupply of $O_2$ and substrate, but there was a marked increase in permeability during the next $\frac{3}{4}$ h, and the difference between the mannitol and inulin spaces continued to increase ($p < 0.025$) through the remainder of the recovery period. As shown by the interrupted line in Fig. 4, tissue water began to increase about 1 h after the rapid increase in membrane permeability, at a time when the membranes had already become very permeable to mannitol. During the latter part of the recovery period, the increase in the mannitol space was accompanied by an increase in the inulin space from $2.56 \pm 0.10$ (S.E.M.) ml/g to $3.40 \pm 0.14$ ml/g ($p < 0.001$). The increase in the inulin space was probably the result of inulin penetration into the intracellular compartment (see below), which would have reduced the difference between the mannitol and inulin spaces and would have obscured somewhat the increased permeability to mannitol calculated from the difference. Figure 5 shows the changes in the various water compartments that occurred during the latter part of the recovery period. Since it is likely that these changes reflected the entry of water into the cells, or the penetration of the extracellular markers, they have been calculated with respect to the volume of intracellular fluid in the normal retina.

**Discussion**

**Relation of Changes in Total Water and Inulin-free Water to Changes in Intracellular Water**

In retinas incubating under control conditions, the inulin space has been found to correspond quite closely to the mannitol space and to the spaces occupied by the readily elutable Na$^+$ and Cl$^-$; so that inulin appears to be a satisfactory label for extracellular water, and the volume of inulin-free water provides a good indication of the volume of intracellular water. In experiments in which the volume of intracellular water has been varied over a wide range by changing medium osmolality, there has been little change in the volume of extracellular water, so that the changes in total water have paralleled closely the changes in cell volume. In the present experiments, there was a close parallelism at first between changes in inulin-free water and changes in total water (cf. first portions of the recovery curves in fig. 3). However, as the post-ischemic retinas were observed over a period of hours, there was an increase in the volume of distribution of inulin, so that the increase in total water exceeded the increase in inulin-free water (cf. latter portions of curves of fig. 3 and also fig. 5). The increase in the inulin space was preceded by an increase in the mannitol space (fig. 4), and these changes probably reflect the penetration, first of mannitol and then of inulin, into damaged cells. Under these circumstances, the increase in total water probably provides a better measure of cell swelling than the increase in inulin-free water, though at some point it becomes a matter of semantics as to whether to include the contents of severely damaged cells in the intracellular or the extracellular phase.

In interpreting measurements of water shifts made on the tissue as a whole, it is necessary to consider the heterogeneity of the different cell types. It is evident from histological examination that cells may be quite different in their susceptibility to ischemic swelling and that even amongst cell types that exhibit considerable swelling there may be individuals that are markedly contracted instead — the "dark-cell" change. In the experiments reported here, the largest increases in tissue water would have represented about a 60% increase in average cell size. In view of the heterogeneity of the histological appearance of retinas that have been treated similarly (ibid), it seems likely that many of the cells had swollen to at least twice their normal size.

**Cell Shrinkage and Cell Swelling in Retinas Subjected to Conditions Simulating Complete Circulatory Arrest In Vivo**

When ECF volume was restricted during the period of $O_2$ and substrate deprivation, a much shorter period...
of deprivation resulted in severe swelling than when ECF volume was large (compare top two curves in fig. 1), suggesting that something in addition to the energy deprivation per se played an important role in the swelling. Quite remarkably, with deprivations slightly longer than those that could be reversibly sustained, there was no evidence of swelling for a prolonged period after the ischemia. Indeed, these retinas showed a significant reduction in inulin-free water when examined ½ h and 1½ h after their return to control medium (fig. 3, lower right). A reduction in cell size is an unexpected consequence of ischemia, but it has also been observed histologically under similar circumstances. Using both light and electron microscopy, Shay (personal communication) observed a large proportion (about one third) of shrunken cells in the photoreceptor and ganglion cell layers when retinas were examined shortly after a period of ischemia just long enough to cause irreversible damage. If the retinas were ischemic for longer, or if they were allowed to "recover" in control medium, there was a progressive reduction in the number of shrunken cells and an increase in swollen cells. Garcia and Kamijo reported both swelling and shrinkage of neurons in primate cortex that had been perfusion-fixed with gluteraldehyde following relatively short periods of middle cerebral artery occlusion. The shrunken cells that appear during ischemia may be related to the "dark" neurons described by neuropathologists. Though "dark" neurons are usually considered an artifact, their contraction may reflect the operation of a similar mechanism. The conditions that caused contraction of retinal cells in the present experiments corresponded to those that caused stiffening of the tissue as a whole. Both responses may reflect a cytoskeletal change, perhaps secondary to the entry of Ca++. Following the initial reduction in tissue water and beginning about 2 hr after the ischemic insult, there was a progressive increase in total water and inulin-free water, and these were still increasing at the end of the 4½ h recovery period (figs. 3 & 5). Measurements of 2-DG uptake in retinas that were similarly treated have shown no change in energy metabolism to account for the delayed swelling: though 2-DG uptake was moderately reduced by the ischemic insult, it showed a progressive improvement during the recovery period to a level of about ¾ of normal. On the other hand, the changes observed in plasma membrane permeability appear to provide an adequate explanation for the swelling. Permeability to mannitol was significantly increased (p < 0.01) after 1½ h of recovery when there was as yet no evidence of cell swelling (fig. 4); and the permeability continued to increase (p < 0.025) during the remainder of the recovery period. As the membranes became increasingly permeable to mannitol, there was indirect evidence of an increased permeability to inulin as well (fig. 5); and it seems reasonable to conclude that at some point in this progression, membrane permeability had increased sufficiently to permit a net influx of electrolytes, and water. The finding of an increased permeability to mannitol before there was an increase in cell volume is consistent with a previous study in which plasma membranes that had been moderately damaged by hypotonic swelling, or by an increase in ionic strength, exhibited an increased permeability to mannitol while still maintaining quite normal electrolyte distributions. Once the point is reached at which there is a net flux of electrolytes and water into the cell, the resulting swelling must compromise further the integrity of the membrane with acceleration of the pathological process.

The alteration responsible for the increased membrane permeability must have been initiated during the ischemic insult and appears to be a consequence of the reduction in ECF, since retinas that were energy-deprived in a large volume of ECF were much less vulnerable and exhibited a different sequence of changes (see below). When cells are energy-deprived in a small volume of ECF, there is a rapid fall in extracellular Ca++. Since Ca++ is required for membrane integrity, it is possible that the changes observed resulted from the combination of low Ca++ and failed energy metabolism. Alternatively, the membranes may have been damaged by toxic substances accumulating in the restricted volume of ECF during the ischemia. For example, arachidonic acid released during the ischemia may be converted during the recovery period to prostaglandins which in turn may cause an increase in membrane permeability. The plasma membrane has been implicated as a primary site of irreversible damage.

Cell Swelling During Energy Deprivation With a Large Volume of ECF

When retinas were deprived of both O₂ and substrate in a large volume, the cell swelling differed in several respects from that observed during deprivation in a restricted volume: (1) a 60% longer deprivation period was required before there was appreciable swelling (fig. 1); (2) the swelling appeared during the deprivation and with no apparent latency; (3) when O₂ and substrate were resupplied there was a reduction in the swelling rather than a progression (fig. 3); (4) there was relatively little increase in plasma membrane permeability, and the increase that was observed could probably be attributed to the swelling rather than to intrinsic damage. The increase in cell water that was measured in these experiments paralleled quite closely the reduction in the cell's capacity to utilize glucose, as assessed in an earlier study by 2-DG uptake: (1) both 2-DG uptake and intracellular water were unaffected by anoxia or substrate deprivation alone, but both were markedly affected by the combined deprivation; (2) there was a prompt, though slight, recovery of both 2-DG uptake and cell swelling when the retinas were returned to control medium; (3) as the duration of the deprivation was increased, the failure of the cells to recover normal volume corresponded fairly closely to their failure to reestablish their glucose metabolism (fig. 6). The extent of the reduction in energy metabolism seems adequate to account for the swelling. For example, following 50 min of deprivation the retinas recovered only 25% of their normal 2-DG uptake and showed a 40% increase in water. In view of the hetero-
Relation of Cell Swelling to Cell Death

When the retinas were examined 4 3/4 h after the ischemic insult, there was a close correlation between loss of viability as assessed by failure to reestablish protein synthesis, and cell swelling as evidenced by an increase in tissue water; and the correlation was maintained when both the nature of the ischemic insult and its duration were varied (fig. 2). Such a correlation would be expected either if cell swelling was the cause of cell death or if cell swelling was a consequence of cell death.

Figure 6. Retinas were deprived of O2 and substrate for 30, 40, or 50 min while immersed in a large volume of electrolyte solution, and then returned to control medium for 4 3/4 h. The increase in total water (interrupted line) is compared with the reduction in 2-DG uptake (solid line), measured under the same conditions in a previous study (4). Bars show S.E.M.

References

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 O2 and substrate. Rabbit retinas were deprived in vitro of both O2 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 O2 and substrate deprivation in order to modify the changes taking place in the interstitial fluid as a result of the failure of energy metabolism. When O2-free, substrate-free extracellular electrolyte solution was added to the retinas to reduce the ischemia-induced changes in the interstitial 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++ 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+ and fall in Ca++); and catabolic products that accumulate in the cells may also appear in the extracellular fluid (e.g., lactic acid and NH3+).

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 O2 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 O2 nor substrate. In the second, exposure to the ischemia-induced changes in the ECF was demonstrated with ion-selective micropipette. Proc Natl Acad Sci USA 74: 1287-1290, 1977.

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