Cerebral Anoxia: An Improved In Vitro Model for Biochemical Study

BY TAKEHIKO YANAGIHARA, M.D.

Abstract: Cerebral Anoxia: An Improved In Vitro Model for Biochemical Study

A previously described in vitro model for cerebral anoxia has been modified to ensure vitality of the rabbit brain slices. The oxygen or nitrogen atmosphere was provided by a continuous gas flow system. Amino acid incorporation into proteins was studied during recovery periods up to 60 minutes. An initial decrease of incorporation and subsequent recovery were observed after an anoxic period of five minutes. On the other hand, a marked decrease and only partial recovery were seen after anoxic periods of 15 minutes or longer. No consistent change was observed in acid-soluble radioactivity. This in vitro model appears to be feasible for various biochemical studies of cerebral anoxia.

Additional Key Words: amino acid incorporation, brain slices, cerebral anoxia

Methods

Albino rabbits (2.5 to 3.0 kg) were anesthetized by intravenous injection of pentobarbital and perfused with ice-cold Ringer's solution through the left ventricle of the heart. Each brain was quickly removed and the cerebral hemispheres were cut into 0.4-mm-thick slices. The slices were divided into control and experimental groups and each group was placed in a 50-ml flask containing the following incubation medium (10 ml/gm of tissue): Tris-HCl buffer (pH 7.4), 38mM; sodium phosphate buffer (pH 7.4), 2.0mM; NaCl, 100mM; KCl, 5mM; MgCl₂, 1.0mM; CaCl₂, 1.8mM; and glucose, 10mM (final pH 7.4 and approximately 290 mOsm). Each flask was capped with a rubber stopper and oxygen was circulated freely into the flask and out through another tube opening under water.

The initial incubation under continuous oxygen flow was carried out for 15 minutes at 37°C with constant shaking at 140 rpm. During this incubation, the tip of the glass tube was kept 5 mm above the surface of the incubation medium. At the end of this period, the flask representing the anoxic condition was switched to nitrogen gas and the glass tube was lowered into the incubation medium for two minutes. The control flask was handled in the identical manner except that oxygen flow was maintained. After two minutes, the tip of the glass tube was raised again to the previous level and incubation was continued for the scheduled period.

At the end of incubation, tissue from each flask was recovered by sieving, divided into four portions, and placed in separate 25-ml flasks containing 5 ml of the same incubation medium as before but with the addition of L-leucine-4,5-³H (Amersham/Searle; adjusted to 100 mCi/mmol) at a concentration of 5 μCi/ml medium. Each flask was thoroughly flushed with oxygen and sealed with a rubber stopper; incubation was continued at 37°C in the shaking water bath. Except for the period of incubation, tissue and incubation medium were handled at 4°C from the time of removal of the brain from the animal.

Flasks were removed at 15, 30, 45, and 60 minutes and one or two brain slices were taken, briefly blotted on filter paper, and weighed. These slices were later homogenized in 1.0 ml of cold 5% trichloroacetic acid (TCA). A supernatant fraction was obtained by centrifugation and used for measurement of TCA-soluble radioactivity. The rest of tissue from each flask...
was diluted to 40 ml with 0.32M sucrose in 10mM Tris-HCl buffer (pH 7.4) and recovered by brief centrifugation. The pellet was homogenized in cold 5% TCA and then centrifuged to recover the pellet which was further washed three times; the third wash was for 15 minutes with 5% TCA at 90°C. The pellet then was extracted with alcohol-ether (1:1) and ether. The dried sample was dissolved in 1.0N sodium hydroxide and an aliquot was taken for protein determination according to Lowry and co-workers.2 Another aliquot was solubilized with Soluene-100 (Packard Instrument Co.) in a counting vial and counted in toluene-based scintillation solution in a Packard Model 3375 or a Nuclear Chicago Mark II liquid scintillation spectrometer. The external standard-channel ratio system was used for quench correction, and the final result was expressed as disintegrations per minute per milligram protein (DPM/mg protein).

For determination of TCA-soluble radioactivity, an aliquot of the TCA supernate was counted in Insta-Gel (Packard Instrument Co.) in the same manner as for protein radioactivity, and the result was expressed as DPM/mg wet tissue weight.

Results

With the present incubation system, the rate of leucine incorporation into protein of brain slices was approximately linear during the interval investigated (table 1). TCA-soluble radioactivity tended to decrease as the duration of the incorporation period increased. Variation among TCA-soluble radioactivities was higher than that of protein radioactivities.

Figure 1 shows the progressive decrease of leucine incorporation during anoxic periods ranging from 3 to 30 minutes. With anoxia for three minutes there was no decrease in leucine incorporation. With anoxia for five minutes, incorporation was slightly decreased during the initial recovery period of 15 minutes. Since the normal variation (standard deviation) between two paired control values was within 15%, the recovery after an anoxic period of five minutes is considered to be complete within 60 minutes of recovery time. After an anoxic period of ten minutes, inhibition of protein synthesis became more definite. It was particularly so during the first 30 minutes of recovery. However, there was a definite tendency toward recovery and, although variation (standard error) was high, there was even nearly complete recovery in some instances. With an anoxic period of seven minutes, the results were between those for five minutes and ten minutes.

Once brain slices were exposed to anoxia for longer than ten minutes, inhibition became more pronounced. Although there was still a tendency toward recovery after anoxic periods of 15 and 20 minutes, it was only partial, and specific radioactivities were only up to 50% of control values even at 60 minutes. After an anoxic period of 30 minutes, leucine incorporation was only 30% of the control value and there was hardly any tendency toward recovery.

Contrary to amino acid incorporation into proteins, TCA-soluble radioactivity did not show any consistent changes (fig. 2). Variances were much higher than in the case of incorporation, but this cannot explain the lack of consistent changes. Comparison of figures 1 and 2 indicates that decrease of amino acid incorporation into proteins was independent of change in amino acid uptake into brain slices.

Discussion

In the previous model,1 one point of questionable validity was the period of anoxia which inevitably occurred between the time of intracardiac perfusion and the time of initiation of incubation. This problem was minimized by using ice-cold Ringer's

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**TABLE 1**

Leucine Incorporation Into Proteins and Leucine Uptake Into Normal Brain Slices*

<table>
<thead>
<tr>
<th>Duration of Incubation (min)</th>
<th>Protein Specific radioactivity (DPM/mg protein)</th>
<th>TCA-soluble Specific radioactivity (DPM/mg wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4,058 ± 207</td>
<td>30,897 ± 1,165</td>
</tr>
<tr>
<td>30</td>
<td>8,032 ± 169</td>
<td>30,374 ± 1,566</td>
</tr>
<tr>
<td>45</td>
<td>11,129 ± 435</td>
<td>24,176 ± 1,955</td>
</tr>
<tr>
<td>60</td>
<td>14,424 ± 530</td>
<td>22,454 ± 1,346</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± SE for eight control values from experiments undertaken for an anoxic period of three minutes.
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solution for perfusion and subsequent maintenance of tissues at 4°C. Indeed, the results from the previous experiments with three-stage incubation suggested that this anoxic period did not jeopardize production of the anoxic condition during subsequent experimental periods. However, it was desirable to clarify this problem further. Another problem was the very low temperature of the brain tissue and incubation medium at the time of inducing the anoxic condition. As Kramer and co-workers have demonstrated, brain tissue tolerates anoxia much longer under profound hypothermia, and adenosine triphosphate (ATP) levels return to normal rapidly afterward. Therefore, with the previous model there would have been some delay in producing the desired anoxic condition before the temperature in the incubation flask reached the equivalent of body temperature.

In the present model, these two problems were taken into consideration and necessary modifications were introduced to eliminate them. The initial incubation of brain slices for 15 minutes under oxygen atmosphere and at 37°C would bring the brain slices to what is considered physiological condition for in vitro study, since the ATP level in brain slices can be restored to 90% of the in vivo level within ten minutes after initiation of aerobic respiration. The temperature inside the flask can reach that of the water bath during the initial incubation. A rapid change from oxygen to nitrogen atmosphere in the flask and in the incubation medium at the initiation of the anoxic condition was incubation medium. Thus, the present experimental incubation medium. Thus, the present experimental model could induce anoxic condition instantaneously.

Another modification was introduced in the composition of the incubation medium. In the previous investigation the incubation medium which was used was chosen because it had been used successfully in the past. However, it was discovered that this incubation medium resulted in a different pattern of leucine incorporation in various subcellular fractions during the second incubation compared to the pattern during the first incubation (unpublished data). Therefore, it was modified to produce similar patterns during the first and second incubations. It also was found that exogenous ATP did not change the extent of inhibition of leucine incorporation, particularly after short anoxic periods (unpublished data). Therefore, ATP was eliminated from the incubation medium.

The decrease of leucine incorporation seen in the present investigation is similar to that seen in the previous one. There was more extensive inhibition after longer anoxic periods in the present study compared to the previous one. There was no consistent change in TCA-soluble radioactivity, as was the case before. Initial inhibition and subsequent recovery were seen during anoxic periods of five to seven minutes and marked inhibition with only partial recovery was seen after anoxic periods of 15 minutes or longer, suggesting that the former is a reversible process and the latter is irreversible. Further investigation is currently in progress, with longer recovery periods, to define the reversibility of the anoxic condition.

Acknowledgment
The author is indebted to Mrs. Rita M. Seghers, Mrs. Karol L. Broadwater, and Mrs. Mary C. Riewe for their careful technical assistance.

References
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Stroke. 1973;4:409-411
doi: 10.1161/01.STR.4.3.409
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1973 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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