A Possible Role of Lipid Peroxidation in Cellular Damages Caused by Cerebral Ischemia and the Protective Effect of α-Tocopherol Administration

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SUMMARY Incomplete global cerebral ischemia was induced by clamping the bilateral common carotid arteries of spontaneously hypertensive rats (SHR) and blood reperfusion was allowed by declamping the arteries after indicated times. To investigate the possible role of lipid peroxidation which causes irreversible ischemic cell injury during ischemia and subsequent reperfusion, cerebral energy metabolism, brain edema, neurological signs and cerebral and serum lipid peroxides were examined. The effect of α-tocopherol administration on these parameters was also studied from the standpoint of its action as a free radical scavenger.

During ischemia up to 5 hours, cerebral ATP decreased and lactate increased rapidly, and concomitantly neurological signs, such as eye closure and jumping seizures, and slowly progressing brain edema were observed. The level of lipid peroxides in the brain and serum remained practically unchanged during ischemia, although an increasing tendency was noted.

When blood reperfusion was allowed 3 hours after ischemia, tissue ATP level was restored only partially (67.4% of normal), but lactate returned to the normal level. The reperfusion resulted in a rapid rise in the lipid peroxide level both in cerebral tissue and serum and also caused a more severe expression of neurological signs.

Intravenous injection of α-tocopherol (20 mg/kg body weight) 30 minutes prior to ligation of the carotid arteries significantly suppressed the rise in lipid peroxides both in the brain and serum, improved the severely expressed neurological signs, and promoted resynthesis of ATP. These improvements in the parameters were observed only after the reperfusion was made following ischemia for 3 hours.

The results suggest that α-tocopherol plays a role in protecting ischemic cellular damages by scavenging free radicals and subsequently lipid peroxides formed by oxygen supply through blood reperfusion.

Cerebral ischemia causes depletion of energy reserves and loss of neuronal functions. At the early stage of ischemia, recirculation of blood flow restores the energy reserves and neuronal functions, but after the critical time of ischemia these functions are restored only partially upon reperfusion. Blood reflux under such conditions is assumed to be more harmful to the organ or tissue than continued ischemia alone.

The mechanism of irreversible cell damage caused by cerebral ischemia has been reviewed.1 Recently several clinical and experimental data have suggested that cellular damage in cerebral ischemia is due to oxidative damage caused by free radical formation and lipid peroxidation,1-7 but no study has been reported in the literature on the detection of lipid peroxidation in vivo after reperfusion following cerebral ischemia.

Preliminary results from our laboratory have indicated that occlusion of both carotid arteries in non-hypertensive rats (NTR) results in a less pronounced fall in carotid back flow pressure and also a less reduction in cerebral blood flow11 than in spontaneously hypertensive rats (SHR), which may cause a smaller decrease in ATP level in NTR than in SHR.9 These results suggest that NTR may tolerate bilateral carotid ligation without difficulty.

In this paper an enhanced formation of lipid peroxides is described in the ischemic brain as well as in serum only after reperfusion following bilateral occlusion of the carotid arteries of spontaneously hypertensive rats (SHR). Pretreatment of SHR with α-tocopherol, an antioxidant,8 suppressed the rise in lipid peroxides after reperfusion, which was accompanied by improvements of energy metabolism, brain edema, and neurological signs. These results are also described in this report.

Materials and Methods

1) Induction of Ischemia and Subsequent Reperfusion

Eighty-six 20-week-old male spontaneously hypertensive rats (SHR) weighing 250-300 g (from Hoshino Experimental Animals Co.) were used. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital at 20-30 mg/kg. Atropin sulfate (0.1 mg) and heparin (100 I.U.) were injected intraperitoneally to prevent vago-reflex and coagulation. Cerebral ischemia was induced by clipping the bilateral common carotid arteries (CCA) with Scoville microclips and this was continued for 1, 3 and 5 hours. Recirculation of blood flow was established by releasing the clips following 3 and 5 hours of ischemia.

Reperfusion was allowed for 3 hours after 3 hours of ischemia and for 1 hour after 5 hours of ischemia. In the latter case, reflow was limited to one hour because of a high mortality rate.
We have previously reported that ischemia of 2 to 3 hours is critical for reversible cerebral damages in 20-week-old SHR.

Until 2-hour ischemia following reflow, the animals did not show any neurological deficits and ATP was restored completely upon reperfusion. After 3 hours of ischemia, however, neurological signs were not improved and ATP could be restored only partially.

Attention was given to the animal’s age (20 weeks) and experimental room temperature (25°C), because these factors affected the severity and reversibility of ischemia. All experimental groups consisted of 5 to 7 rats.

2) Administration of α-tocopherol

α-tocopherol was injected at 20 mg/kg body weight in the femoral vein 30 minutes before starting ischemia. As placebo, a solvent consisting of a detergent was injected intravenously in the same volume as the sample.

3) Measurement of Cerebral ATP and Lactate

At various indicated times, the animals were decapitated and placed in liquid nitrogen and tissue ATP and lactate concentrations were measured as described previously.

Cerebral hemispheres were chiselled out rapidly and stored at −80°C for several days until analysis. The tissue was weighed and homogenized at −40°C with Polytron 20ST (Kinematica, Swiss) in 20 ml of a solution containing cooled (−40°C) 8% perchloric acid and 40% ethanol. The homogenate was centrifuged at 15,000 g for 10 minutes at −20°C. The pellet was diluted with 1 N NaOH for assay of protein contents by the method of Lowry et al. The supernatant was neutralized with K₂CO₃ and centrifuged at 15,000 g for 10 minutes at −20°C to remove the precipitates. ATP concentration in the supernatant was determined by the enzymatic fluorometric method and lactate by the standard enzymatic method using lactate dehydrogenase.

4) Measurement of Lipid Peroxides

The brain tissue was frozen and chiselled out according to the same procedure. Lipid peroxides were determined with thiobarbituric acid (TBA) by a modified method of Masugi and Nakamura (1976). There are several methods to determine lipid peroxides. The method used for measurement of TBA-RS as MDA has been verified as a method protecting degradation of intact polyunsaturated fatty acids by solubilizing with sodium dodecyl sulfate (SDS). TBA-reactive substance (TBA-RS) was measured fluorometrically as malondialdehyde (MDA).

The modified procedure includes 1) addition of 10 mM ethylene diamine tetraacetate (EDTA) to the homogenizing medium to chelate Fe²⁺ and 2) homogenization of the tissue under N₂ stream to protect in vitro lipid peroxidation. Serum lipid peroxides were determined by the method of Yagi. Student’s t-test was used in the statistical analysis.

Results

1) Changes in Energy Metabolites

In the control group, cerebral ATP depleted remarkably to 0.73 ± 0.36 μmol/g wet weight (28% of the control) at 3 hours of ischemia, critical time of ischemia, and further decreased gradually until 5 hours (fig. 1 and table 1). When reperfusion was allowed after 3 hours of ischemia, ATP level was restored to 1.78 ± 0.10 μmol/g wet weight (67% of the control) within 1 hour and remained at a steady level thereafter.

 Pretreatment with α-tocopherol did not improve the ATP depletion during ischemia but stimulated significantly the recovery of ATP after 3 hours of recirculation following 3 hours of ischemia (fig. 1).

Lactate concentrations in the brain increased gradually during the first hour after ischemia and then rapidly to a level 3 and 6 times higher than the non-ischemic level after 3 and 5 hours of ischemia, respectively. The elevated lactate level after 3-hour ischemia returned to the control level upon recirculation in both the placebo group and α-tocopherol administered group. No significant difference was observed between the two groups in the changes of lactate level (fig. 2 and table 1). The accumulated lactate in the brain tissue might be partially washed out in the circulating blood upon reflow. This may be the reason for the lack of a significant difference in lactate levels between the two groups.

FIGURE 1. Effect of Vit. E on the ATP level in the brain of SHR after ligation of bilateral common carotid arteries and reflow of circulation. Values are means ± SEM. Significant differences are indicated (Student’s t-test); versus placebo group ***; p < 0.001.
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4. Lipid Peroxides in the Brain and Serum

With reflow after 5 hours of ischemia, 50% (4/8) became comatous and 50% (4/8) died of apnea.

α-tocopherol administration did not improve the exhibited neurological signs during ischemic periods up to 5 hours. However, during reflow periods after 3 hours of ischemia, eye closure decreased to only 23% of the animals and jumping seizures disappeared completely. During the reflow period after 5-hour ischemia, the neurological signs became severer than those at 5-hour ischemia and 25% of the animals died of apnea, although a mild deterioration of these signs was observed in α-tocopherol-treated groups when compared to the placebo group.

3) Brain Edema

In the placebo group, protein concentrations decreased significantly at 3 and 5 hours of ischemia, indicating edematous state of the brain, which further progressed for 1 hour upon reflow. Reflow for 3 hours following 3 hours of ischemia restored the protein concentration to the normal level (fig. 3).

In the α-tocopherol administered group, the decrease in protein concentration during ischemia was smaller but not significantly so than the placebo group. The protein concentration after 1 hour of reflow following 3 hours of ischemia was significantly higher than in the placebo group (fig. 3), and then it recovered to the control level after 3 hours of reflow. These results indicate prevention of edematous changes in the reflow period and suggest that no breakdown of cerebral protein was evident at least during 3-hour ischemia and following reflow period.

2) Neurological Signs (table 2)

Neurological deficits were observed exactly for each time interval as shown in table 2 during ischemia or following reperfusion.

In the placebo group all animals exhibited neurological signs of eye closure during 3 hours of ischemia and jumping seizures were observed in 32% of the cases (6 animals out of 19).

When ischemia was prolonged to 5 hours, jumping seizures developed in all animals and 30% (4/13) of them became comatous.

With reflow after 3 hours of ischemia, neurological signs persisted almost identically to those in 3-hour ischemia except one developed apnea.

With reflow after 5 hours of ischemia, 50% (4/8) became comatous and 50% (4/8) died of apnea.

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4) Lipid Peroxides in the Brain and Serum

In the placebo group, TBA-RS in the brain tissue increased gradually during 5 hours of ischemia. Reflow allowed after 3 and 5 hours of ischemia rapidly and significantly increased the tissue TBA-RS in 1 hour to 2.73 ± 0.31 and 2.97 ± 0.35 nmol/mg protein, respectively (table 3).

Pretreatment with α-tocopherol, on the other hand, suppressed the gradual increase in TBA-RS level during 3 hours of ischemia.

TABLE 1

Effect of Vit. E on the ATP, Lactate and Protein Contents in the Brain of SHR after Ligation of Bilateral Common Carotid Arteries and Reflow of Circulation

<table>
<thead>
<tr>
<th>Ischemia (hour)</th>
<th>ATP (μmol/g wet wt.)</th>
<th>Lactate (μmol/g wet wt.)</th>
<th>Protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Vit. E</td>
<td>Placebo</td>
</tr>
<tr>
<td>0 0</td>
<td>2.64 ±0.18</td>
<td>2.91 ±0.16</td>
<td>7.38 ±0.85</td>
</tr>
<tr>
<td>1 0</td>
<td>2.65 ±0.24</td>
<td>2.74 ±0.28</td>
<td>9.53 ±0.62</td>
</tr>
<tr>
<td>3 0</td>
<td>0.73 ±0.36†</td>
<td>0.86 ±0.41†</td>
<td>23.0 ±3.3*</td>
</tr>
<tr>
<td>1 1</td>
<td>1.78 ±0.10†</td>
<td>1.84 ±0.23†</td>
<td>10.6 ±0.6*</td>
</tr>
<tr>
<td>5 0</td>
<td>0.25 ±0.05†</td>
<td>0.51 ±0.35‡</td>
<td>45.0 ±2.33‡</td>
</tr>
<tr>
<td>1 1</td>
<td>0.74 ±0.13‡</td>
<td>1.09 ±0.13‡</td>
<td>13.8 ±2.69</td>
</tr>
<tr>
<td>3 3</td>
<td>1.83 ±0.30*</td>
<td>1.20 ±0.01‡</td>
<td>9.86 ±1.69</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Significant differences are indicated (Student's t-test); versus non-ligated group (0-hour group).

*p < 0.05; †p < 0.005; ‡p < 0.001 versus placebo group; §p < 0.05; ¶p < 0.001.
The increase in TBA-RS level found after reflow following 3-hour ischemia was significantly smaller than in the placebo group (fig. 4).

However, pretreatment failed to suppress the rise of TBA-RS level detected during reflow following 5-hour ischemia.

Changes in serum TBA-RS level during ischemia and subsequent reflow periods in the placebo group showed patterns nearly identical to those found in tissue TBA-RS (fig. 4 and 5). Serum TBA-RS level increased gradually during ischemia up to 5 hours and rapidly and significantly after reflow following 3 and 5 hours of ischemia (fig. 5).

α-tocopherol administration could significantly suppress the rise in serum TBA-RS detected especially after reflow following 3 and 5 hours of ischemia.

**Discussion**

Several recent clinical and experimental data have suggested that cellular damage in cerebral ischemia is at least partly due to oxidative damage caused by free radical formation and lipid peroxidation. This is partly supported by the finding that transient incomplete ischemia is more deleterious for brain tissue than complete cessation of cerebral blood flow.

On the other hand, reversible brain ischemia has given rise to reduction in tissue concentration of reduced glutathione without a reciprocal increase in oxidized glutathione. This result fails to give support to the hypothesis that peroxidative damage occurs during or following ischemia.

Free radical formation during cerebral ischemia but not after reperfusion has been shown indirectly by a progressive decrease in the amount of ascorbic acid, a normally occurring antioxidant and free radical scavenger.
The foregoing results do not give any conclusive evidence that oxidative damage is or is not a cause of cellular damage in cerebral ischemia. Lipid peroxidation in cerebral ischemia was observed in this study particularly when oxygen supply was restored in the tissue by reperfusion (fig. 4). To avoid lipid peroxidation in vitro during the process of TBA reaction, 10 mM EDTA was added to the homogenization buffer to chelate Fe\(^{2+}\) and the assay procedures were carried out under 100% N\(_2\) as described in Methods.

The increase in lipid peroxides, especially detected after restoration of blood flow following 3 hours of ischemia, is assumed to be due to free radicals formed during this period. This is supported by the fact that pretreatment with a-tocopherol, a free radical scavenger,\(^8,^{21}\) suppressed the rise in lipid peroxides especially in the post ischemic period (fig. 4).

This pretreatment concomitantly stimulated recovery of ATP (fig. 1), reduced the appearance of neurological signs (table 2), and improved brain edema (fig. 3), after restoration of blood flow but not during ischemia. It was considered that a-tocopherol would protect irreversible ischemic cell injury during ischemia and following reflow quenching free radicals but not energy deterioration and disturbed neuronal function caused by insufficient blood flow. Thus, a-tocopherol was only effective in the reflow period outwardly.

The principal role of α-tocopherol as an antioxidant is to neutralize free radicals which could initiate a chain reaction particularly in highly unsaturated lipids, resulting in the formation of peroxides and products of their subsequent degradation.\(^{21,22}\)

The results obtained by administration of α-tocopherol, especially suppression of the rise in lipid peroxides in the tissue as well as in serum in the post-ischemic period, are compatible to the hypothesis that a reduction in capacity for natural defense against oxidative damage may initiate deleterious oxidative reactions induced by the sudden increase in tissue oxygen tension upon re-establishment of circulation. Lipid peroxidation as a possible cause of post-ischemic cerebral injury was proposed in the gerbil brain ischemia.\(^7\)

In similar ischemic experiments with the rat liver, a marked increase in lipid peroxide formation as malondialdehyde was detected in the tissue as well as in mitochondria only after reperfusion following warm ischemia but not during ischemia.\(^{23,24}\) This rise in lipid peroxidation was completely inhibited by administration of coenzyme Q\(_{10}\)^{23,24} acting as an antioxidant.\(^{25}\) This treatment was accompanied by stimulation of ATP recovery and by preservation of such mitochondrial functions as respiratory control index and ADP/O ratio.\(^{23}\) These results may support the assumption obtained in cerebral ischemia with α-tocopherol pretreatment.

After ischemia of 5 hours and subsequent reflow, α-tocopherol pretreatment showed no protective effect on damaged cellular functions as well as on the rise in tissue lipid peroxide level (fig. 4 and 5 and table 1). This suggests that ischemia of 5 hours and post-ischemic reflow cause irreversible damages, to which α-tocopherol administration is no longer effective.

Changes in lipid peroxides in the serum reflect nice-
ly those found in the tissue (fig. 4 and 5), suggesting an easy transport of tissue lipid peroxides into serum. It is therefore of significant value clinically to measure serum lipid peroxides.

References


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Stroke. 1983;14:977-982
doi: 10.1161/01.STR.14.6.977

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