Protection of Rabbit Retina From Ischemic Injury by Flupirtine

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Purpose. The aim of this study was to determine whether flupirtine can slow down the changes seen in the rabbit retina after ischemia–reperfusion.

Methods. A suction-cup procedure, which raises intraocular pressure, was used to give an ischemic insult to the rabbit retina. Electroretinograms were recorded before ischemia and at different periods after ischemia. In some instances, flupirtine was injected into the eye before ischemia. Immunohistochemistry was used to study the effect of ischemia–reperfusion on the γ-aminobutyric acid (GABA) immunoreactivity and uptake of serotonin by the retina. The effect of flupirtine and ischemia on retinal adenosine triphosphate (ATP) levels were determined in in vivo and in vitro experiments.

Results. Ischemia for 75 minutes causes a change in the nature of normal GABA immunoreactivity and a reduction in the b-wave of the electroretinogram. When flupirtine is injected into the vitreous humor at the onset of ischemic insult, the changes in GABA immunoreactivity are reduced and the recovery of the reduced b-wave of the electroretinogram after defined reperfusion times is enhanced significantly. Rat retinas incubated in vitro in physiological solution containing flupirtine caused a significant rise in the tissues' ATP content compared with control samples. However, incubation of the tissue in physiological solution saturated with nitrogen caused a drop in retinal ATP levels. Addition of flupirtine prevented this decrease from taking place. Serotonin injected into the vitreous humor of the rabbit eye is taken up by certain amacrine cells. The amount of serotonin taken up is reduced greatly in retinas, as judged by immunohistochemistry when tissues are subjected to ischemia. Because the ischemia was shown to cause a drop in the tissue ATP level, it is concluded that this is the cause of the reduced uptake of exogenous serotonin. Injection of flupirtine into the vitreous humor during ischemia enhanced the uptake of serotonin.

Conclusions. Combined data show that flupirtine is a neuroprotective agent in retinal ischemia and that one mode of its mechanism of action is to influence ATP levels. Flupirtine may lower the activity of NMDA receptors, thus causing ATP levels to be less affected in the presence of the drug as a secondary effect. Invest Ophthalmol Vis Sci. 1996;37:274–280.

been postulated that ischemia leads to a loss of cellular homeostasis and a shortage of available adenosine triphosphate (ATP).1,3 This causes a release of glutamate and other mediators, which results in a rapid cellular efflux of potassium and influxes of sodium, calcium, and chloride with obligated wa-

ter.1,3,4 The neuronal damage is probably secondary to the influx and mobilization of calcium and/or activation of a variety of enzymes, such as protein kinase C, proteases, and lipases.1,3,6

Glutamate N-methyl-D-aspartate (NMDA) receptors are thought to play a major role in the damaging effects elicited by an ischemic insult because the activation of these receptors results in an influx of calcium.1,3,5 Moreover, electrophysiological studies show that NMDA channel activity is influenced by physiological levels of ATP, with ATP preventing transition of the receptor to the "run-down" state.7,8 This suggests that in a cell, reduced ATP levels, caused by ischemia, reduce the opening of the NMDA channel. The implication of these data is that the opening of the NMDA

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channel pore caused by glutamate can be challenged either by reducing intracellular levels of ATP or by antagonists acting at the receptor level.

Flupirtine is a centrally acting nonopoid analgesic, and it does not appear to interact with either serotonin, dopamine, nicotine, or adrenergic receptors. Flupirtine does, however, decrease the spinal polysynaptic flexor reflex mediated by NMDA receptors. Moreover, flupirtine antagonizes the NMDA-induced changes on $\gamma$-aminobutyric acid (GABA) in the retina and protects against neurotoxicity induced by NMDA or by GP120, the human immunodeficiency virus coat protein. However, recent binding studies failed to show that flupirtine has an affinity for the various NMDA recognition sites (unpublished data, 1995), suggesting that the substance has a unique pharmacologic action.

Ischemic injury to the retina is a major cause of visual loss and morbidity. Clinically, retinal ischemia is recognized by an alteration in the b-wave of the electroretinogram (ERG), cotton wool spots, pathologic "cupping" in the optic disc, and rupture of retinal blood vessels. Experimental studies show that when the retina is subjected to an ischemic insult, glutamate is released, and subsequent damage can be nullified by treatment with the NMDA antagonist dextromethorphan. The aim of the current study was to determine whether flupirtine acts in a similar way to dextromethorphan. A report has appeared to show that flupirtine enhances the recovery of the b-wave of the electroretinogram of rats subjected to transient occlusion of their carotids. Because the procedure for inducing retinal ischemia in that study does not lead to detectable pathologic changes in the retina, results can be interpreted as well to show that flupirtine enhances ocular blood flow and that, therefore, by implication rather than by demonstration, it is a neuroprotective agent.

METHODS

All investigations involving animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult albino rabbits bred in our laboratory (each weighing 1.5 to 2 kg) were kept on a 12-hour light–12-hour dark cycle. A rabbit was placed in a restrainer box, and 3 ml of a solution of Rompun (0.1 ml/kg; Bayer Pharma, Sens, France) and Vetranquil (0.4 ml/kg; Sanofi Santé Nutrition Animale, Libourne, France) in 0.9% NaCl was injected into the ear vein. Urethane (50% urethane in 0.9% NaCl) was then injected intraperitoneally at a concentration of 0.5 ml/kg. The animal was still conscious after this form of anesthesia but was sedated enough for the experiments to be performed.

Ischemia Experiments

After topical instillation of 1% Tropicamide (Smith and Nephew, Chauvin, UK) to an anesthetized animal, a suction cup was placed on the cornea of one eye, and the pressure was reduced to 500 mm Hg. This reduction of pressure causes the intraocular pressure to rise to approximately 120 mm Hg, thus inducing ischemia. The raised intraocular pressure was maintained for 75 minutes before removing the suction cups to allow reperfusion to take place or killing the animal with a lethal injection of sodium pentobarbital.

In all experiments, immediately after the induction of ischemia, 10 $\mu$L of solution containing 10 mM flupirtine (estimated volume of vitreous humor in the eye is 1 ml; therefore, the approximate concentration of flupirtine in the eye is 100 $\mu$M), or solvent was injected into the vitreous humor of the eye. The flupirtine solution and solvent (a mixture of gluconic acid and sodium hydrosulphite) were from ASTA Medica (Frankfurt, Germany).

In some experiments, 45 minutes after the initiation of ischemia, the eye was injected once again with 10 $\mu$L solution containing either solvent or 10 mM flupirtine base plus 0.1 mM serotonin. At this stage, the other (control) eye received 10 $\mu$L solution containing only 0.1 mM serotonin. Ischemia was allowed to continue for 30 minutes more.

Electroretinography

In these experiments, animals were dark adapted for at least 30 minutes. A platinum recording electrode was placed on the cornea, a reference electrode was connected to the tongue, and a ground electrode was connected to the ear. A stimulus frequency of 0.5 Hz was provided by a strobe placed 15 to 20 cm in front of the animal. Fifteen consecutive responses were amplified and averaged using a 1902 Signal Conditioner/1401 Laboratory Interface (CED, Cambridge, UK). The b-wave amplitude of electroretinograms was measured from the troughs of the a-waves to the peaks of the b-waves. In this limited study, no attempt was made to analyze the oscillatory potential or the c-wave or a-wave of electroretinograms.

Immunohistochemistry

Tissues fixed in 2% paraformaldehyde were cryopreserved in 30% sucrose, and frozen retinal sections (10 $\mu$m) were cut from retinal pieces approximately 5 mm from the optic nerve and were mounted on gelatine-coated glass slides. Sections were then processed for the localization of GABA and serotonin as described elsewhere. Primary antibodies used were monoclonal anti-serotonin (1:200; Dako, Copenhagen, Denmark) and polyclonal anti-GABA (1:400; Cambridge Research).

FIGURE 1. The relative effect of flupirtine on adenosine triphosphate (ATP) levels in the rat retina. It can be seen that flupirtine significantly \((P < 0.05; \text{Student's } t\text{-test})\) increases the basal (control) level of tissue ATP, whereas the solvent (placebo) had no effect. When retinas were incubated in the absence of oxygen, the tissue ATP level dropped \((P < 0.01; \text{Student's } t\text{-test})\), but this reduction was much reduced in the presence of flupirtine. Results represent mean ± SEM.

Adenosine Triphosphate Experiments
A spectrophotometric procedure was used to measure ATP levels.\(^{22}\) To show that the suction-cup procedure to induce retinal ischemia is effective, one eye of each animal was given an insult for 90 minutes while the other eye served as a control. Animals were killed immediately with sodium pentobarbital, retinas were dissected rapidly, and their ATP content was determined.

In other experiments, rats were decapitated, their retinas were dissected, and a single retina was incubated in 2 ml physiological Locke’s solution for 10 minutes at 37°C. The Locke’s solution lacked magnesium and contained (in mmol/l) 157Na\(^{+}\), 5.6K\(^{+}\), 2.3Ca\(^{2+}\), 164.2Cl\(^{-}\), 3.6HCO\(_3^{-}\), 5 HEPES, and 5.6 glucose. The solution was equilibrated before use with O\(_2\)/CO\(_2\) (95%/5%), and it had a pH of 7.2. Twenty microliters of either water, solvent, or solvent containing 100 \(\mu\)M flupirtine was then added to samples, and the incubation was continued for 20 minutes. These experiments were duplicated, but, on this occasion, the incubation solution was infused constantly with nitrogen to displace oxygen from the solution. Retinas were analyzed immediately for ATP content and were related to the retinal protein content as determined by the procedure of Bradford.\(^{23}\)

RESULTS

Influence of Ischemia and Flupirtine on Retinal Adenosine Triphosphate Content
Table 1 shows results from two different experiments in which 90-minute ischemia by the suction-cup procedure reduced the retinal ATP content by approximately 50% compared with the control eye.

Rat retinal tissue incubated for 20 minutes with 100 \(\mu\)M flupirtine caused a highly significant increase in the tissue’s ATP content (Fig.1). Of note, the solvent did not alter tissue ATP levels significantly. When the incubation solution was infused with nitrogen, control and solvent tissue ATP levels were significantly reduced, whereas flupirtine treatment now had little influence on the normal basal ATP level.

Influence of Flupirtine on the b-wave of the Electroretinogram
To show the effect of flupirtine, the b-wave recovery was analyzed 2 hours and 2 days after reperfusion relat-

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TABLE 1. Effect of 75-minute Ischemia by the 'Suction Cup' Procedure on ATP Content in Rabbit Retina

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATP Content (nmol/mg protein)</th>
<th>% Change Compared With Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>68.6</td>
<td></td>
</tr>
<tr>
<td>Ischemia 1</td>
<td>33.3</td>
<td>51.4%</td>
</tr>
<tr>
<td>Control 2</td>
<td>55.3</td>
<td></td>
</tr>
<tr>
<td>Ischemia 2</td>
<td>31.9</td>
<td>42.5%</td>
</tr>
</tbody>
</table>

Data are from two animals in which the retina from the fellow eye served as a control sample. ATP = adenosine triphosphate.

FIGURE 2. Effect of ischemia with reperfusion time of 2 hours and 2 days on the b-wave of the electroretinogram of the retina in the absence of flupirtine (black bars) and the presence of flupirtine (striped bars). It can be seen that the b-wave does not change in the control eye (open bars). Ischemia followed by 2 hours of reperfusion shows that the b-wave is reduced almost completely, but after 2 days there is still almost no recovery (black bars). In contrast, the b-wave of retinas that received flupirtine during and after ischemia was reduced to a lesser extent 2 hours after reperfusion and showed significant \((P < 0.05; \text{Student’s } t\text{-test})\) recovery after 2 days (striped bars). Results represent mean ± SEM; \(n = 5\).
FIGURES 3. The influence of ischemia for 75 minutes on γ-aminobutyric acid (GABA) immunoreactivity in the rabbit retina. Retinal sections shown in A and B and then C and D are for two different rabbits, respectively. (A, C) Normal GABA immunoreactivity in the control eyes of the two rabbits. GABA “staining” is associated with amacrine cell bodies (arrowheads) and terminals in the inner plexiform layer (large arrow). Ischemia (B) plus solvent treatment causes a change in the nature of the GABA immunoreactivity, with fewer immunoreactive cell bodies and three bands of immunoreactivity (small arrows) in the inner plexiform layer. In contrast, the GABA immunoreactivity in retinas delivered an ischemic insult in the presence of flupirtine (D) showed only slight changes. Scale bars = 30 μm.

Influence of Flupirtine on the Ischemic-induced Changes in GABA Immunoreactivity

The GABA immunoreactivity in rabbit retinas given an ischemic insult for 75 minutes by the suction-cup procedure for 75 minutes showed a change from the control tissue. The GABA “staining” in the inner plexiform layer showed banding rather than homogeneity (see Figs. 3A, 3B). In addition, the number of immunoreactive GABA perikarya was reduced because of ischemia, although this was variable. When flupirtine was injected into the eye, there were fewer ischemia-induced changes in the retinal GABA immunoreactivity than in the retina of an animal that also underwent ischemia but was placebo treated (Figs. 3C, 3D).

Influence of Flupirtine on Serotonin Uptake During Ischemia

Results depicted by Figures 4A and 4B were typical when both eyes were injected with serotonin but only one eye underwent ischemia for 75 minutes (n = 5). Serotonin was injected into both eyes 30 minutes before the end of the ischemic insult. It can be seen that the amount of serotonin immunoreactivity associated with the retina that underwent ischemia (Fig. 4B) was greatly reduced compared with the control retina (Fig. 4A). When serotonin was injected in an eye 75 minutes after ischemia and that eye was left for a reperfusion time of 30 minutes before analysis, both the retina subjected to ischemia and the control retina (n = 3) took up serotonin to the same degree (results not shown).

Retinas from eyes of animals that underwent ischemia and received serotonin but were treated with flupirtine (n = 12; see Methods for details) displayed an enhanced uptake of serotonin compared with retinas that underwent ischemia but were treated with
FIGURE 4. This figure shows data from three different rabbits. In each case, one eye was subjected to ischemia and was treated with flupirtine or solvent (see Methods for details). Both eyes were injected with serotonin. (A,B) One eye of one rabbit served as a control (A), whereas the other eye of the same rabbit received ischemia plus solvent (B). (A) It can be seen that in the control eye, serotonin is taken up by amacrine cell bodies (large arrows) and terminals (small arrows). (B) Very little serotonin, seen as a faint band in the inner plexiform layer (small arrow), is taken up by the retina that received ischemia plus solvent. (C to F) Sections from retinas of two rabbits. Identical experiments were carried out on the two rabbits to give similar results. In these experiments, the eye subjected to ischemia also was injected with flupirtine (D,F). Results show that flupirtine enhanced the uptake of serotonin in retinas that underwent ischemia (D,F), although the degree of uptake of serotonin varied between experiments. Scale bars = 30 μm.

DISCUSSION

Results demonstrate that flupirtine acts as a neuroprotective agent against retinal ischemia. This conclusion is based on the finding that flupirtine enhanced the recovery of the b-wave of the electroretinogram and counteracted the changes observed in the GABA immunoreactivity caused by ischemia. In a previous study on rats, flupirtine was found to stimulate the recovery of the b-wave of the electroretinogram in which ocular blood flow was reduced by transient occlusion of the carotids. However, occlusion of the carotids does not lead to detectable histologic damage to the retina. By definition, a neuroprotective agent is one that protects against neuronal damage so it is desirable to use a model system in which damage does occur. The suction-cup procedure for inducing retinal ischemia causes morphologic retinal changes to occur 4 to 6 days after reperfusion (unpublished data, 1995). Thus, the demonstration that reduction in the b-wave is attenuated by flupirtine when ischemia is induced by the suction-cup procedure provides convincing evidence that flupirtine is a neuroprotective agent, and it corroborates the study on the rat retina.

It is known that ischemia-induced morphologic damage to the retina and alteration of the b-wave of the electroretinogram are reduced by the NMDA antagonist, dextromethorphan. Moreover, glutamate has been shown to be released from the retina by...
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ischemia. Also, our previous findings have shown that the ischemic-induced changes in the GABA immunoreactivity can be nullified by glutamate antagonists. All these data argue in favor of the idea that the glutamate released during ischemia is a major cause of ischemic damage. The finding here that flupirtine counteracts the changes in GABA immunoreactivity caused by ischemia suggests that flupirtine also acts as a glutamate antagonist and, hence, is neuroprotective.

Certain studies have pointed to flupirtine as an NMDA antagonist. For example, flupirtine decreases the spinal polysynaptic flexor reflex mediated by NMDA receptors. In in vitro preparations, flupirtine protects against neurotoxicity induced by NMDA or GP120, the human immunodeficiency virus coat protein, as well as the NMDA-induced changes in GABA immunoreactivity in the retina. However, results of our binding studies have failed to show that flupirtine has any affinity for the glutamate recognition site, glycine site, magnesium site, polyamine site, or the phenylcyclidine site of the NMDA receptor (unpublished data, 1995). Nevertheless, it cannot be excluded that the substance has an affinity for yet another site on the NMDA receptor complex—for example, the Zn binding site (see ref. 27). It also has been proposed that the NMDA receptor can be modulated by the redox state, so flupirtine could have an action here.

Our current finding that flupirtine enhances tissue ATP levels presents another possible way in which the substance might protect tissues against damage. Both in vitro and in vivo studies on rat and rabbit tissues, respectively, clearly show that flupirtine enhances basal levels of ATP. Moreover, “starving” the tissue of oxygen (by bubbling nitrogen) in vitro reduces retinal ATP levels, but when flupirtine is present, this reduction is counteracted almost completely. Flupirtine, therefore, appears to stimulate ATP production in some way. This is particularly interesting given the fact that the avascular rabbit retina is largely dependent on glycolytic metabolism rather than on the citric acid cycle, the principal route for glucose metabolism in the vascular rat retina.

It generally is accepted that in ischemia, ATP is exhausted because of a lack of oxygen and that when this occurs, a cascade of events leading to cell damage is initiated. Clearly, any substance that stimulates ATP availability, as flupirtine appears to do, will have a “neuroprotective” role. Interestingly, there is evidence that the rundown of the NMDA receptor can be prevented by ATP, suggesting that when the intracellular level of ATP is enhanced, the “opening” of the NMDA channel pore caused by an agonist is elevated. Thus, a substance that raises the ATP levels theoretically would function not unlike that of a NMDA agonist. But this would be contrary to the neuroprotective action of flupirtine as well as to the observations that flupirtine inhibits the activity of NMDA receptors. One possible way of explaining the current data is as follows. In the presence of flupirtine, the lower activity of the NMDA receptors would reduce the cytotoxic [Ca]; subsequently, ATP levels would be less affected as a secondary effect (i.e., it is the increase in the [Ca], that is responsible for lowering the ATP levels of the cell). It is well known that Ca reduces the capacity of the mitochondria to synthesize ATP.

Previous studies have shown that serotonin uptake by the rabbit retina is dependent on ATP. The finding that retinal tissues given an ischemic insult by the suction-cup procedure have a reduced ability to accumulate exogenous serotonin suggests that the ischemia has been effective in reducing tissue ATP levels. During reperfusion, the tissue rapidly regenerates ATP levels because serotonin is taken up immediately during reperfusion (results not shown). The current findings also show that 45 to 75 minutes of ischemia reduce, but do not exhaust, ATP levels because some serotonin is still taken up. This is confirmed by ATP analysis showing that the suction-cup procedure for inducing ischemia reduces ATP levels by only approximately 47%. The animals also appear to respond in a variable way to the same insult. Thus, the amount of serotonin taken up by retinas subjected to ischemia is always reduced but is not consistent. Nevertheless, a comparison of results from individual animals demonstrated clearly that flupirtine enhanced the uptake of serotonin during ischemia. This provides additional evidence that the substance raises ATP levels. The actual mechanism of action of flupirtine may be, as already suggested, to lower the activity of the NMDA receptors; therefore, as a secondary effect, ATP levels would be less affected in the presence of the drug.

Key Words
flupirtine, protection, rabbit, retinal ischemia

References
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