Flunarizine Reduces Cerebral Infarct Size After Photochemically Induced Thrombosis in Spontaneously Hypertensive Rats

J. Van Reempts, B. Van Deuren, M. Van de Ven, F. Cornelissen, and M. Borgers, DSc

The cerebroprotective effect of flunarizine was studied in a minimally invasive model of photochemically induced cerebral infarction in spontaneously hypertensive rats. Intravenous administration of the photosensitizing dye rose bengal and intense focal illumination of the brain produced a deep cortical infarction that resulted from singlet oxygen-induced peroxidative injury to the endothelial membrane, subsequent platelet adhesion, and eventual thrombus formation. The infarct size was calculated from area measurements on consecutive histologic sections prepared from the brain cortex 4 hours after the onset of the insult. Oral treatment with 40 mg/kg flunarizine 3 hours before photoexcitation resulted in a significant reduction of the median infarct size from 11.75 mm$^3$ in the untreated group to 6.40 mm$^3$ in the treated group ($n = 13$, $p < 0.001$). At this dose, flunarizine had no effect on systemic blood pressure. In a separate experiment the area of thrombotic obstruction was quantified 30 minutes after the onset of light exposure. Flunarizine did not significantly reduce early thrombus formation (2.28 mm$^3$ in the untreated and 1.78 mm$^3$ in the treated group) ($n = 12$, $p = 0.2$). The infarcted area at 4 hours was considerably larger than the initial thrombotic area. Protection with flunarizine against development of cortical infarction has been unequivocally shown. Although some effect may already be present at the early stage of lesion formation, the major protective action admittedly occurred in the later postinsult period when the lesion was expanding. The observed beneficial effects may be attributed to preservation of the integrity of endothelial cell membranes (reduction of platelet adhesion and vasogenic edema formation), of neuronal cell membranes (inhibition of toxic Ca$^{2+}$ overload), and of glial cell membranes (prevention of cytotoxic edema formation).

The results indicate that flunarizine may be of clinical use for the suppression of thrombotic stroke.

(Stroke 1987;18:1113-1119)

In stroke prevention, attention is given not only to primary treatment of risk factors such as hypertension but also to the secondary prevention of atherothrombotic cerebral infarction. Ca$^{2+}$-mediated phenomena may play a major role in the evolution of cerebral damage. For this reason the use of Ca$^{2+}$ entry blockers as a therapeutic measure to prolong neuronal cell survival or to reduce neuronal vulnerability has become the subject of widespread research. Few animal models of focal cerebral ischemia are available for the pathologic and pharmacologic study of ischemic stroke. Recently Watson et al. developed a minimally invasive method of inducing cerebral infarction after photochemically initiated thrombosis in the rat cortex. This attractive method is based on photodynamic generation of singlet molecular oxygen from systemically injected rose bengal after excitation with a beam of green light transmitted through the intact skull. Peroxidative damage to the endothelial membrane provides the initial stimulus for platelet adhesion and eventual thrombus formation.

The present study evaluated the effects of flunarizine, a selective blocker of pathologic Ca$^{2+}$ influx. Recently published papers report beneficial effects of this drug in models of global ischemia and graded hypoxia-oligemia. The presently used dose of flunarizine was selected according to its absorption and tissue distribution characteristics in rats and with respect to its activity in other ischemia studies. In a pilot study, using a slight modification of the photochemical infarct model of Watson et al., we obtained significant protection with flunarizine in normotensive rats. Systemic hypertension is a recognized risk factor for the development of stroke in humans. Moreover, spontaneously hypertensive rats (SHR) have more severe and reproducible lesions after middle cerebral artery occlusion as well as after photochemically induced thrombosis (our initial observations). Therefore, SHR were used in the present study. In addition, the experimental set-up was simplified by the use of a standard fiber-optic light source. Infarct size was assessed 4 hours after light excitation using routinely processed histologic sections. At this time, integrity of the infarcted tissue was still sufficiently preserved, which facilitated preparation of intact sections for quantitative measurements. Early thrombus formation and areas of increased blood–brain barrier (BBB) permeability were visualized by continuous i.v. infusion of Evans blue. The results obtained with flunarizine will be discussed with respect to its presently known pharmacologic profile.
Materials and Methods
Animal Preparation and Treatment
Fed male SHR (Okamoto strain) weighing 250–270 g were used in this experiment. All rats were adapted to the laboratory environment for at least 1 week. One group of 13 rats was treated orally with 40 mg/kg flunarizine suspended in vegetable oil 3 hours before the insult. A concurrent group of 13 rats served as untreated controls. A separate group of 5 sham-operated rats served as unchallenged controls. Tracheal intubation was performed under 4% halothane in a mixture of 70% N₂O and 30% O₂. During the subsequent surgical procedures halothane was maintained at 1%. The left femoral artery and vein were catheterized for monitoring mean arterial pressure (MAP), for blood sampling, and for drug delivery. A scalp incision was made to expose the skull, and the rats were subsequently placed in a stereotactic apparatus. After disconnection of halothane, the rats were immobilized by repeated i.v. injections of 1 mg succinylcholine in 0.2 ml saline, and artificial ventilation was started with a N₂O:O₂ (70:30) mixture (Stephan respirator V, Nordstedt, F.R.G., ventilation rate 70/min, inspiration pressure ±1,200 Pa). A steady-state period of 30 minutes was allowed for blood gases to reach normal values, i.e., Paco₂ 35–48 mm Hg and pH 7.35–7.45.

Induction of Thrombotic Infarction
Rose bengal (7.5 mg/ml in 0.9% NaCl) was infused i.v. over 2 minutes (0.133 ml/100 g body wt, final concentration 10 mg/kg). When the infusion was completed, illumination was started with a Schott KL 1500 fiber-optic light source (Mainz, F.R.G.) equipped with an Osram Xenophot 150-W lamp. The lamp was replaced once during the study. No special filter setting was used. The fiber was placed in a cylindric mount containing a 1-mm diaphragm at its base. The holder was attached to the stereotactic apparatus and positioned over the rat's right hemisphere 3 mm lateral to the midline and 5 mm anterior to the interaural line. Light exposure lasted 20 minutes and was performed after replacement of the halogen lamp (see above). The rats were then perfusion-fixed, and consecutive 100-μm sections were prepared. Evans blue, left in the fiber-optic light source (Mainz, F.R.G.) equipped with an Osram Xenophot 150-W lamp. The lamp was replaced once during the study. No special filter setting was used. The fiber was placed in a cylindric mount containing a 1-mm diaphragm at its base. The holder was attached to the stereotactic apparatus and positioned over the rat's right hemisphere 3 mm lateral to the midline and 5 mm anterior to the interaural line. Light exposure lasted 20 minutes. Sham-operated rats did not receive rose bengal but were similarly irradiated. At the end of the experiment, the catheters were removed and all wounds were sutured with wound clips. The rats were kept under a heating lamp until spontaneous respiration resumed.

Morphologic Analysis of Infarct
Four hours after the onset of illumination, the rats were anesthetized with ether and transcardiacaally perfused with Karnovsky’s fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde. Serial 100-μm Vibratome (T.P.I., St. Louis, Mo.) sections, prepared at 100-μm intervals, were routinely stained with azure A–eosin B at pH 4.5. Some of the intermediate sections were postfixed in OsO₄, embedded in Epon, and cut at 2-μm intervals for detailed light microscopy. The infarcted area was measured in each 100-μm section with a Quantimet Q970 automatic image analyzer (Cambridge, U.K.). The volume of the infarct was calculated by numerical integration according to the trapezoidal rule:

\[ V = \left(2t \sum_{i=1}^{n} A_i\right) - \frac{t(A_1 + A_n)}{2} \]

where \( V \) = volume in mm³, \( t \) = section thickness in mm, and \( A \) = infarct area in mm².

Assessment of Early Thrombus Formation
To measure the initial thrombotic area, 2 groups of 12 rats, prepared and treated as described above, were continuously infused with 2% Evans blue in 0.1 M PO₄ buffer. Dye infusion started at the onset of illumination and lasted 30 minutes (total injected volume, 1 ml). Light exposure lasted 20 minutes and was performed after replacement of the halogen lamp (see above). The rats were then perfusion-fixed, and consecutive 100-μm sections were prepared. Evans blue, left in the microvessels due to blood stasis in the thrombotic area or extravasated toward the cerebral parenchyma due to increased vascular permeability, could be recognized either by fluorescence microscopy or by densitometry. Volumes were measured and calculated as described above.

Statistical Analysis
All data were expressed as median values with their 95% confidence limits, based on the sign test. Paired data (infarct size) were compared with the Wilcoxon test. Unpaired data (thrombus size, infarct vs. thrombus size) were compared with the Mann-Whitney U test. Two-tailed \( p \leq 0.05 \) was considered significant.

Results
Physiologic variables (MAP, arterial blood gases) recorded just before the onset of illumination are summarized in Table 1. Except for a significantly lower Paco₂ in the treated group, no differences were detected between control and flunarizine groups.

At 4 hours after the insult, all sham-operated rats remained completely free of damage. In the flunarizine

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<th>Table 1. Physiologic Variables Before Induction of Thrombotic Cerebral Infarction in Control and Flunarizine-Treated Rats</th>
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<td>Group</td>
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<td>Controls</td>
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Values are median and 95% confidence limits. 40 mg/kg flunarizine administered p.o. 3 hours before insult.

* \( p < 0.01 \) by two-tailed Mann-Whitney U test.
and control groups, however, clearly delineated spherical lesions were found (Figure 1). Due to slight acidophilia in the infarcted area (Figure 2a) the lesion could be easily recognized in coronal sections. Pronounced vascular congestion was found in the center of the lesion. Analysis of 2-μm Epon sections (Figure 3a) revealed clear signs of platelet thrombus formation, perivascular edematous swelling, and focal hemorrhages. Many perivascular spaces contained lipid-like material. Neuronal and glial cells were irreversibly damaged. The neuropil showed granular disintegration and in certain areas was abundantly filled with plasma-derived amorphous material. Whereas the surrounding tissue in 100-μm sections appeared normal (Figure 2a), in 2-μm sections a peripheral rim was found, characterized by severe astrocytic edema but with open vessels and normal neurons (Figure 3b). Cortical damage depended highly on intensity of the emitted light. As can be derived from Table 2, infarct size increased almost threefold after replacement of the halogen lamp. In the control group, the median infarct size was 11.75 (3.9-14.08) mm³ (Figure 4). Oral treatment with 40 mg/kg flunarizine 3 hours before the insult resulted in a significant reduction (46%) of the infarct size to 6.4 (2.6-9.59) mm³ in the treated group (n = 12, p < 0.001). The total (leaky + thrombotic) volume measured 4.13 (2.75-5.23) and 3.41 (1.38-4.35) mm³ in the untreated and treated groups, respectively (n = 12, p = 0.4).

Comparison of the infarct size at 4 hours with the size of thrombosis at 30 minutes showed a significantly larger infarcted volume in both untreated and treated groups (p < 0.001 for each group). Likewise, the infarct size was significantly larger than the total volume

<table>
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<tr>
<th>Experimental condition</th>
<th>Infarct size (mm³)</th>
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<tr>
<td>Illuminated before (Series A)</td>
<td>Controls</td>
</tr>
<tr>
<td>3.58</td>
<td>3.08</td>
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<tr>
<td>3.38</td>
<td>1.95</td>
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<tr>
<td>3.90</td>
<td>3.68</td>
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<tr>
<td>5.27</td>
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<td>Illuminated after (Series B)</td>
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<tr>
<td>13.06</td>
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Figure 2. Azure–eosin-stained 100-μm coronal sections through cortex of rats sacrificed 4 hours after photochemical induction of thrombotic infarction. Lesions appear well-demarcated as result of acidophilic staining in infarcted area; surrounding cerebral tissue remains unaltered. In control rats (a) large infarcted area reaches corpus callosum (CC), which becomes locally displaced by expanding lesion (arrow). Oral pretreatment with 40 mg/kg flunarizine 3 hours before ischemic insult (b) results in smaller but morphologically similar infarction.

Discussion

The light/dye model of Watson et al proved to be a suitable model to study pharmacologic protection against cerebral infarction. Previous experience with this model has revealed that more reproducible and larger infarcts were produced in SHR (unpublished results), possibly due to a genetic or acquired problem of inadequate collateral circulation in SHR. The present study demonstrates the protective action of flunarizine against development of focal cerebral infarction in SHR. The results confirm our earlier findings in normotensive rats.

Flunarizine is a selective Ca²⁺-overload blocker with no or only low affinity for slow Ca²⁺ channels. The compound has been previously tested in different animal models of cerebral ischemia and hypoxia. Amelioration of ischemic damage occurred even after postischemic treatment and could not be correlated with an improvement of postischemic cerebral flow. Prevention of toxic Ca²⁺ overload by direct stabilization of the neuronal membrane may offer an explanation for the good outcome of flunarizine-treated animals after hypoxic or ischemic insults. Although the pathophysiologic mechanisms leading to cerebral necrosis in the present stroke model may be somewhat different, it is not excluded that Ca²⁺ at certain stages of the injurious process also plays an important role.

A radial expansion at the margins of the initial thrombotic core at between 30 minutes and 4 hours, as shown by Dietrich et al, could also be observed in the present study. Increased vascular permeability, as in-
Evans blue extravasation, was seen at 30 minutes at the periphery of the ischemic zone. This suggests the presence of an area at risk. Indeed, it is not excluded that the vascular endothelium in the peri-thrombotic territory underwent sufficient light exposure to become labile. Although platelet adhesion had not yet occurred, vasogenic edema in this supposedly hyperemic area might well induce secondary ischemia.

Flunarizine significantly reduced infarct size after 4 hours. At 30 minutes only a slight reduction of platelet thrombosis could be observed. Although the presence of a protective effect from the onset of insult may not be excluded, the present data are not sufficient to support this idea. The exact mechanism by which flunarizine exerts its protective action is unclear. It has been shown by Borgers et al that flunarizine protected isolated myocytes from light/dye-induced necrosis in an in vitro model independent of flow variations. Changes in flow profile caused by the drug are thus not likely to be a mechanism by which it reduces infarct size. However, a direct protective effect at the level of the cell membranes of platelets, erythrocytes, and endothelial cells, as well as of neuronal and glial cells, might offer a more plausible explanation. Stabilization of platelet membranes might be sufficient to prevent adhesion and aggregation, be it by alteration of surface receptors or by prevention of Ca entry. However, flunarizine has been shown not to affect platelet behavior since these cells preferentially use intracellular Ca stores and hence do not strictly require transmembrane fluxes for their release reaction. Prevention of Ca entry into red blood cells by flunarizine on the other hand may lead to increased deformability of these cells.
Effects of 40 mg/kg flunarizine p.o. on thrombotic obstruction, changes in vascular permeability, and development of infarction in the cerebral cortex of rats. Flunarizine administered 3 hours before injection with rose bengal and photoexcitation. Thrombotic area and total volume (thrombotic + leaky area) were measured at 30 minutes (n = 12), whereas infarct size was measured at 4 hours after the insult (n = 13). Values are medians and are compared using the Mann-Whitney U test (thrombotic, total) or the Wilcoxon test (infarcted).

and therefore may slow the process of vascular congestion in the excited area. Direct protection of the endothelial membrane offers a more reasonable explanation for the beneficial action of flunarizine in the present stroke model. In previous studies it has been shown that flunarizine protects against endothelial damage induced by citrate, CaCl₂, or lactate in rats, against microvascular permeability increase in rat skin, against atherosclerotic plaque formation in electrically stimulated rabbit carotid artery, and against arachidonic acid-induced damage of the pulmonary endothelium and subsequent platelet thrombosis in mice. The above-mentioned protective effects have been largely attributed to the Ca²⁺-antagonistic effects of flunarizine either by preserving the capacity of the membrane to bind Ca²⁺ or by preventing the noxious transmembrane fluxes of Ca²⁺. In addition, a preservation of the structural integrity of the endothelial membrane may be important to prevent formation of vasogenic edema, which in this model may lead to secondary microvascular compression and hence to an enlargement of the primary ischemic focus.

Finally, it is also important to consider a possible protective effect directly at the level of the cerebral parenchymal cells. This may be expected since flunarizine readily penetrates the BBB. In previous studies we showed a correlation between blockade of subcellular Ca²⁺ overload and structural preservation of neurons after ischemic and hypoxic insults. The direct neuronal effect can also be derived from the study of Höller et al. These authors measured extracellular Ca²⁺ under conditions of oxygen shortage and demonstrated a pronounced delay in cellular Ca²⁺ influx in flunarizine-treated rats. Ashton showed improved posthypoxic recovery of synaptic activity in hippocampal slices from animals pretreated with flunarizine, and Wibo et al demonstrated that flunarizine inhibited K⁺-stimulated Ca²⁺ uptake in rat brain synaptosomes.

Besides neuronal protection, it may also be important to preserve the integrity of the astrocytic compartment. Astrocytes play an important role in modulating neuronal metabolic events and are thought to influence the permeability characteristics of cerebral microvessels. Our light microscopic observations have shown severe astrocytic swelling at the periphery of the thrombotic core, which coincided with increased permeability for Evans blue. Glial changes may thus precede BBB breakdown, and radial expansion of the lesion can be explained by either mechanical compression of microvessels due to swelling of glial processes.
or by secondary formation of mural thrombi in the marginal area.

In several of the above-mentioned studies, important differences have been observed between various subclasses of Ca\textsuperscript{2+} blockers. Class IV Ca\textsuperscript{2+} antagonists (for classification see Reference 28) generally were superior in their protective action, suggesting that mechanisms other than slow channel blockade are responsible for the prevention of pathologic transmembrane Ca\textsuperscript{2+} fluxes.

Although further investigations are required to elucidate the brain-protective mechanisms of flunarizine, this and several other experimental studies may justify its clinical use in the mitigation of thrombotic stroke.

Acknowledgments

The authors are grateful to Dr. D. Ashton and Dr. F. De Clerck for reviewing the manuscript, to Mr. L. Wouters for performing statistical analysis, to Mr. G. Jacobs for preparing the illustrations, and to Mrs. S. De Cauwer for typing the manuscript.

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*Stroke*. 1987;18:1113-1119
doi: 10.1161/01.STR.18.6.1113

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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