Protective Effect of Flunarizine on Blood–Brain Barrier Permeability Alterations in Acutely Hypertensive Rats

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Background and Purpose: Increased cerebrovascular permeability to protein is a well-documented finding in acute and chronic hypertension. In this study, we examined the effect of pretreatment with a calcium entry blocker, flunarizine, on the increased cerebrovascular permeability to protein that develops in norepinephrine-induced acute hypertension.

Methods: Protein transfer was assessed qualitatively with Evans blue dye and quantitatively with iodine-125–labeled serum albumin.

Results: Brains of hypertensive rats showed increased permeability to both tracers. The number and size of the areas of Evans blue extravasation were smaller in the hypertensive groups pretreated with flunarizine intravenously. This was supported by the quantitative studies, which demonstrated a significant decrease in protein transfer in total brain of hypertensive rats pretreated with intravenous flunarizine, 1 mg/kg (p<0.005) and 2.5 mg/kg (p<0.001). Data from individual brain regions showed that pretreatment with flunarizine resulted in significant reduction of protein transfer in most brain regions.

Conclusions: These data support the hypothesis that calcium plays a role in increased cerebral endothelial permeability in hypertension. (Stroke 1991;22:1265–1269)
hypertension (HTD T1), b) 1 mg/kg (HTD T2), and c) 2.5 mg/kg (HTD T3) i.v. 3 minutes before the induction of hypertension.

Rats were injected intravenously with 10 μCi RISA (ICN Biomedicals Canada Ltd, Montreal, Quebec) in 0.3 ml saline. Ten minutes later, 1 ml 2% Evans blue dye was injected intravenously followed 5 minutes later by induction of hypertension by intravenous norepinephrine (5 μg/0.5 ml saline) infused for 2 minutes. Blood (0.3 ml) was collected 6, 15, and 23 minutes after administration of RISA for the measurement of plasma radioactivity. Eight minutes after the onset of hypertension the experiments were terminated by an intravenous bolus of sodium pentobarbital (37.5 mg). Control rats were infused with saline instead of norepinephrine, but were otherwise subjected to the same experimental procedures as the test animals.

At the termination of the experiment, a thoracotomy was performed, and 0.9% saline was perfused for 10 minutes via a cannula in the ascending aorta to remove RISA from the lumen of cerebral vessels. Brains were rapidly removed and divided into six regions (Figure 1). In the case of the cerebral hemispheres, brain blocks from both sides were pooled. The areas designated as lobes included both the grey and white matter. The latter was cut away from the areas designated as cortex. Brain and blood samples were weighed, and radioactivity was determined in a gamma counter. The sum of the radioactivity in the six regions was used to calculate the leakage of RISA in the whole brain.

The amount of leakage of RISA into brain tissue was expressed as a percentage of plasma radioactivity, as described previously9 using the following formula:

$$\frac{\text{cpm/mg brain}}{\text{cpm/µl plasma}} \times 100 = \% \text{ protein transfer}$$

Values of the mean percent protein transfer in the whole brain, in individual brain regions, blood pressures, pH, and blood gas values of rats in the different experimental groups were compared simultaneously by the one-way analysis of variance and the Tukey post hoc test.10 Values from these analyses were used to calculate the critical differences between the percent protein transfer in the individual brain regions in the experimental groups. In addition, percent protein transfer results were compared using the Wilkinson repeated-measures analysis of variance,10 in which correlation between the different brain regions is incorporated. Results were accepted as significant at $p<0.05$.

In most series,2,11 only 70–80% of hypertensive animals develop increased permeability to Evans blue. In order not to bias results in favor of a protective action of flunarizine, only animals showing Evans blue extravasation in the hypertensive groups were included in this study.

Results

The resting mean arterial blood pressures of the normotensive controls and the non-drug-treated hypertensive rats were 136±3 and 137±5 mm Hg, respectively. Immediately following the onset of the norepinephrine infusion a rise in blood pressure occurred and at 30 seconds the blood pressure of the hypertensive non-drug-treated group was 224±4 mm Hg (Table 1). The blood pressure reached resting levels in about 5 minutes.

The resting mean arterial pressures of the three drug-treated groups, HTDT1, HTDT2, and HTDT3, were 130±6, 135±4, and 142±3 mm Hg (Table 1.) A transient fall in blood pressure of 8 mm Hg was observed in the two groups of rats that received intravenous flunarizine. The duration of the blood pressure fall was 30 seconds in two rats in the

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<td>Time (min)</td>
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The number of animals in each group is shown in parentheses. Values are mean±SEM. Measurements are millimeters of mercury. Statistical analysis did not show significant differences in the blood pressure levels of the hypertensive (HT) and the three flunarizine-treated hypertensive groups (HTDT1, HTDT2, HTDT3) ($p>0.05$).
FIGURE 2. Mean percent protein transfer in the whole brain of rats in the different experimental groups is shown. The bars denote mean±SEM. There is a significant increase in protein transfer in brains of hypertensive rats as compared with the normotensive controls. Pretreatment with intraperitoneal flunarizine did not have a significant effect on protein transfer. Intravenous flunarizine significantly reduced protein transfer in brains of hypertensive rats (*p<0.005; **p<0.001).

HTDT2 group and 41±7 seconds in the HTDT3 group. Thirty seconds after the onset of the norepinephrine infusion, the mean blood pressures of rats in the HTDT1, HTDT2, and HTDT3 groups were 222±5, 216±3, and 220±7 mm Hg, respectively. Thus, peak blood pressures were not different in the hypertensive non–drug-treated and drug-treated groups.

The mean blood pressure of the HT group was higher than that of the HTDT3 group by 12 and 19 mm Hg at 1 and 2 minutes, respectively (Table 1). Statistical analysis indicates that the observed differences are not significant (p>0.05).

The pH and blood gases of the experimental groups were in the normal range and no appreciable differences in mean values were detected between the groups.

Hypertensive rats showed six to 12 areas of Evans blue extravasation, most frequently in the parasagittal and occipitotemporal cortex, which represent the boundary zones of the areas of supply of the major cerebral artery territories. These areas of extravasation measured 1–2 mm in diameter but rarely were large diffuse areas of extravasation encountered in the occipitotemporal cortex. The number and size of these areas of Evans blue extravasation was less in the drug-treated hypertensive rats particularly those receiving intravenous flunarizine. Such rats showed only two or three areas of Evans blue extravasation. Six of the 26 hypertensive rats did not demonstrate Evans blue extravasation and were excluded from this study.

The mean percent protein transfer in total brain was 0.15±0.01 in normotensive controls and 0.38±0.04 in hypertensive non–drug-treated rats (Figure 2). Thus hypertensive rats showed a significant increase (p<0.001) in mean percent protein transfer in brain. Data from individual brain regions (Table 2) showed that the increase in permeability in hypertensive rats was 100% more than comparable values in controls with increases of about 200% of control values in the occipital lobes and cerebellum.

The mean percent protein transfer in total brain of hypertensive rats pretreated with intraperitoneal flunarizine was 0.31±0.02, a value not significantly different from that of the non-drug-treated hypertensive rats. However, the data of the individual brain regions showed a significant decrease in mean

<table>
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<th>Table 2. Percent Protein Transfer in Individual Brain Regions of Control and Hypertensive Rats</th>
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<td>Pretreatment with flunarizine</td>
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<td>2.5 mg/kg i.v. (5)</td>
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<tr>
<td>Critical difference</td>
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<tr>
<td>Frontal lobe 0.13±0.01 0.30±0.05* 0.18±0.02 0.15±0.03 0.17±0.01 0.12</td>
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<tr>
<td>Frontal cortex 0.21±0.03 0.43±0.07* 0.27±0.04 0.22±0.02 0.25±0.01 0.17</td>
</tr>
<tr>
<td>Occipital lobe 0.13±0.01 0.38±0.03* 0.55±0.03* 0.37±0.04* 0.21±0.02 0.12</td>
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<tr>
<td>Occipital cortex 0.22±0.03 0.42±0.05* 0.34±0.05 0.20±0.02 0.25±0.04 0.17</td>
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<tr>
<td>Cerebellum 0.15±0.01 0.49±0.10* 0.25±0.04 0.21±0.02 0.23±0.02 0.21</td>
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<tr>
<td>Pons 0.22±0.02 0.43±0.08* 0.21±0.03 0.21±0.02 0.24±0.02 0.17</td>
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The number in parentheses indicate the number of animals in each group. Values are mean±SEM. Critical difference is the minimum value needed for significance between any two means (p<0.05).

*Significantly different from the control group. Comparisons without a critical difference were not significant (F test, p>0.05).
percent protein transfer in all regions except the occipital lobes (Table 2).

The mean percent protein transfer in total brain in groups HTDT2 and HTDT3, which were pretreated with intravenous flunarizine, were 0.26±0.02 and 0.22±0.01, respectively (Figure 2). This represented a significant decrease in mean percent protein transfer when compared with the hypertensive non-drug-treated group. The data for the individual brain regions (Table 2) showed that both groups pretreated with intravenous flunarizine demonstrated a significant reduction in percent protein transfer in most brain regions which had values that were similar to those of the normotensive controls (p>0.05).

The results obtained from the one-way analysis of variance by region were confirmed using the repeated-measures analysis of variance in which correlation between regions was taken into account (F=3.215, df=20,54, p<0.001).

Discussion

This study supports previous observations that increased protein transfer occurs in brain in acute hypertension. In addition, this study demonstrates that pretreatment of hypertensive rats with flunarizine results in a significant reduction in protein transfer in most brain regions.

Hypertensive rats showed a high incidence of Evans blue extravasation in the occipitotemporal cortex, which represents the boundary zone between the anterior, middle, and posterior cerebral artery territories. This localization of Evans blue extravasation was similar to previous reports that have emphasized the boundary zone localization of these lesions. Previous studies have demonstrated that only 70–80% of hypertensive rats develop increased permeability to Evans blue or horseradish peroxidase. Similar results were obtained in the present study.

Hypertensive rats showed a significant increase in protein transfer in whole brain (p<0.001). Data from individual brain regions demonstrated that the percent protein transfer was 100% more than control values with increases of about 200% occurring in the occipital lobes and cerebellum. Increased protein transfer in norepinephrine-induced acute hypertension has been well documented by others using RISA9 or horseradish peroxidase 4 as tracers.

The calcium entry blocker used in this study was flunarizine, a diphenylpiperazine with a selective action in arteries. It is reported to be a selective blocker of pathological Ca2+ influx with no or only low affinity for slow Ca+ channels. The dose of flunarizine used in this study is similar to those reported to be effective in rodents in reducing ischemia and hypoxia and endothelial cell injury in vivo.

Studies of noncerebral vessels have reported a reduction or inhibition of vascular permeability in response to flunarizine. Flunarizine inhibited the increased endothelial permeability to horseradish peroxidase that occurred in arteriosclerotic plaques in response to electrical stimulation of the carotid arterial wall by implanted electrodes. Another report described suppression of atherogenesis in rabbit aortas following administration of oral flunarizine. In addition, flunarizine was reported to inhibit in a dose-related manner the enhanced vascular permeability in rat skin induced by histamine, serotonin, bradykinin, and arachidonic acid.

Flunarizine had a protective effect in this study resulting in a reduction in cerebrovascular permeability alterations in most brain regions that had percent protein transfers not significantly different from those of control rats. An exception was the occipital lobes, which included the hippocampus, an area with vessels that lack blood–brain barrier characteristics and normally leak horseradish peroxidase. Reduction in permeability in this region was only achieved by the highest intravenous dose used. Although there are no reports in the literature regarding the effect of flunarizine on cerebrovascular permeability, reduction in size of cerebral infarcts have been observed following pretreatment with flunarizine or isradipine. Although speculative, it is possible that this observed reduction in size of infarcts in these models is due to reduction of cerebrovascular permeability and edema mediated by the calcium entry blockers used.

Flunarizine is reported to produce only slight decreases of systemic arterial blood pressure. This is supported by the findings of the present study in which a fall of 8 mm Hg was observed for periods varying from 10 to 60 seconds so that the blood pressure of animals was restored to resting levels about 2 minutes before the induction of hypertension. The reduction in endothelial permeability produced by flunarizine in this study cannot be attributed to its blood pressure lowering effect since the mean blood pressures of the hypertensive group and the three drug-treated hypertensive groups were not statistically different. Rather, the effect of flunarizine on cerebral endothelial permeability must be related to its known action as a selective Ca2+ blocker in smooth muscle and endothelial cells in conditions where the influx of this ion is stimulated.

Previous studies have demonstrated reduced activity of the cerebral endothelial plasma membrane Na+,K+ and Ca2+-ATPase in permeable arterioles in acute hypertension. Reduced activity of the plasma membrane Na+,K+-ATPase could result in sodium accumulation within the endothelial cell, which would decrease the efficiency of the Na/Ca exchanger and lead to intracellular Ca2+ accumulation. Inhibition of the plasma membrane Ca2+-ATPase would further contribute to increase in intracellular calcium. Possibly, this increase in intracellular calcium mediates increased endothelial permeability.

An alternative suggestion is that a generalized membrane defect may be present in hypertension which could lead to high Na+, low K+, and high intracellular Ca2+ by virtue of leaks across the plasma membrane.
membrane. Since we are dealing with an acute hypertension model, such changes occur transiently when the blood pressure is elevated.

The occurrence of inhibition of Na\(^+\),K\(^+\)-ATPase in hypertension is well documented.\(^{27,30-32}\) The protective action of flunarizine in this study may also be mediated by its effect on this enzyme since flunarizine has been reported to prevent the reduction of Na\(^+\),K\(^+\)-ATPase activity which occurs in cerebral ischemia. The latter was observed in gerbils with ischemia produced by 60-minute bilateral clamping of the carotid artery followed by 40 minutes of reperfusion.\(^7\)

This study supports the hypothesis that Ca\(^{2+}\) is involved in increased endothelial permeability in hypertension. Further studies directed to demonstration of intracellular calcium levels in permeable vessels in acute hypertension will be required to prove this hypothesis.

Acknowledgments

Thanks are expressed to Dr. Paul Gross, Director of the Neurosurgical Research Unit, for valuable suggestions throughout this work and to Dr. Terry Smith, Director of the Queen’s Stat Laboratory, for help with the statistical analyses. The excellent technical assistance provided by Mrs. Verna Norkum and Mrs. Christa Roberts is gratefully acknowledged. Flunarizine was a generous gift of Janssen Pharmaceutica Inc., Mississauga, Ontario, Canada.

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KEY WORDS: blood-brain barrier • calcium channel blockers • hypertension • rats
Protective effect of flunarizine on blood-brain barrier permeability alterations in acutely hypertensive rats.
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doi: 10.1161/01.STR.22.10.1265

*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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