Immunohistochemical Localization of Prostaglandin $F_{2\alpha}$ in Reperfused Gerbil Brain

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Abstract

Immunohistochemical localization of prostaglandin (PG) $F_{2\alpha}$ synthesized during recirculation following experimental forebrain ischemia was studied in Mongolian gerbils. The bilateral carotid arteries were clamped for 5 minutes, and the brains were frozen *in situ* after 5 minutes of recirculation. Sham-operated gerbils not subjected to arterial occlusion served as controls. Cryostat sections containing dorsal hippocampus $10\mu\text{m}$ in thickness were incubated in rabbit anti-PGF $_{2\alpha}$ serum and stained with avidin-biotin-peroxidase complex after fixation in carbodi-imide and Zamboni's solution. In specimens from control animals, blood vessels were faintly stained with anti-PGF $_{2\alpha}$ serum, while neurons and glial cells were less intensely stained or unstained. Specimens from reperfused gerbils demonstrated strongly positive staining for PGF $_{2\alpha}$ in blood vessels, neurons (especially hippocampal pyramidal neurons), and glial cells. Staining for PGF $_{2\alpha}$ in the reperfused animals was substantially diminished by pretreatment with indomethacin (10 mg/kg, i.p.). The results indicate that recirculation after forebrain ischemia results in an increase in PGF $_{2\alpha}$ in neurons, glial cells, and blood vessels.

Key words: prostaglandin, ischemia, brain reperfusion

Introduction

Bilateral carotid artery occlusion in Mongolian gerbils leads to a rapid increase in the amount of free arachidonate released from membrane phospholipids in the brain.³³⁾ During subsequent reperfusion of the brain, the arachidonate can be metabolized to prostaglandins (PGs) or leukotrienes by either a cyclo-oxygenase or a lipoxygenase. Gaudet *et al.*⁷⁾ showed that the brain concentrations of PGD $_2$, PGE $_2$, PGF $_{2\alpha}$, thromboxane B $_2$, and 6-keto-PGF $_{1\alpha}$ were markedly elevated during the early phase of recirculation following forebrain ischemia in gerbils. The results of recent studies^{3,6,12,19,26)} suggest that these PGs may be involved in the pathophysiological consequences of brain ischemia through regulation of cerebral blood flow, vascular permeability, and neurotransmission. Elucidation of the role of PGs requires identification of the specific tissues and cells that synthesize PGs.

In the study presented here, we succeeded in localizing PGF $_{2\alpha}$ synthesis during reperfusion after forebrain ischemia in Mongolian gerbils. For this purpose we used immunohistochemical techniques, specifically, the avidin-biotin-peroxidase complex (ABC) method.¹¹⁾

Materials and Methods

Adult male Mongolian gerbils (*Meriones unguiculatus*) weighing 60–70 gm were anesthetized with 2% halothane in 70% nitrous oxide and 30% oxygen, and the body temperature was maintained at 37°C with a heating blanket. The bilateral carotid arteries were exposed at the neck by means of a microsurgical technique and were occluded for 5 minutes with aneurysm clips (Sugita's temporary clip, type 07-940-51). The halothane was then withdrawn and the experiments were conducted under nitrous oxide anesthesia. After 5-minute occlusion, the clips were removed to allow recirculation. Fourteen animals were sacrificed by freezing *in situ* after 5 minutes of reperfusion. Six of these animals had been

given 10 mg/kg indomethacin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) intraperitoneally 30 minutes prior to arterial occlusion. Eight control animals underwent a sham operation in which the common carotid arteries were exposed but not occluded.

The brains were removed while still frozen and kept at -80°C until immunohistochemical analysis was performed. Coronal sections containing dorsal hippocampus 0.5–1.0 mm posterior to the most rostral tip of the hippocampus were cut into 10- μm sections in a cryostat. The sections were first fixed for 20 minutes in 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide (Sigma Chemical Co.) 50% (W/V) in distilled water on ice at 4°C . They were then fixed in Zamboni's solution for 10 minutes at 4°C . The immunohistochemical staining for $\text{PGF}_{2\alpha}$ was accomplished by the ABC method.

The brain sections were incubated in normal goat serum (Sigma Chemical Co.) for 20 minutes at room temperature, then exposed to rabbit anti- $\text{PGF}_{2\alpha}$ serum (East-Acres Biologicals, Southbridge, Mass., U.S.A.; Bio-Yeda Ltd., Rehovot, Israel) diluted to 1:200 or 1:400 with normal goat serum at 4°C overnight. Biotinylated antibody (Vector Labs, Burlingame, Calif., U.S.A.) diluted to 1:200 with phosphate-buffered saline (PBS) (pH 7.4) was applied to the sections for 30 minutes at room temperature. After blockage of endogenous peroxidase activity with hydrogen peroxide (0.3% in methanol for 30 minutes), the sections were incubated in an ABC complex solution (Vector Labs) in PBS for 45 minutes at room temperature. Finally, the reaction was developed for 1–3 minutes in a fresh preparation of 10 mg of 3,3-diaminobenzidine tetrachloride in 20 ml of 0.05 M Tris-buffered saline (pH 7.6) contain-

ing 0.01% hydrogen oxide. The sections were washed in cold PBS between each procedure. The specimens were counterstained with a hematoxylin solution, dehydrated with increasing concentrations of ethanol followed by xylene, placed under coverslips, and examined under a light microscope.

Specificity of immunohistochemical staining was tested with normal rabbit serum (Sigma Chemical Co.) and with rabbit anti- $\text{PGF}_{2\alpha}$ serum in which $\text{PGF}_{2\alpha}$ was absorbed, according to the method of Perez and McGuckin.¹⁸⁾ The cross-reactivities in this study were 100% for $\text{PGF}_{2\alpha}$, 60% for $\text{PGF}_{1\alpha}$, and $<0.1\%$ for other PGs.

Results

In gerbils not subjected to vessel occlusion, only faint rabbit anti- $\text{PGF}_{2\alpha}$ serum staining was observed in the walls of pial and intraparenchymal vessels and intravascular components (Fig. 1). Some control animals, however, showed no staining of vessel walls. Staining was slight or negative in the intraparenchymal cells of the parietal cortex, hippocampus (Fig. 2), and thalamus.

The reperfused gerbil specimens stained intensely for $\text{PGF}_{2\alpha}$ in vessel walls, neurons, and glial cells (Figs. 3, 4A, and 5). Hippocampal pyramidal neurons were the most deeply stained (Figs. 3B and 5A). At higher magnification, the $\text{PGF}_{2\alpha}$ -positive cells showed intracytoplasmic staining, which spared the nucleus (Fig. 5A). Granule cells in the dentate gyrus were less intensely stained and often only partially stained (Fig. 3C). The neurons of the parietal cortex and thalamus were also positive for $\text{PGF}_{2\alpha}$ (Fig. 5B), although less intensely so than those of the hippocampal pyramid. Glial cells, especially those of

Fig. 1 Immunohistochemical localization of $\text{PGF}_{2\alpha}$ in a hippocampal section from a normal gerbil. Pial and intraparenchymal vessels in the parietal cortex (A) and thalamus (B) are faintly stained with anti- $\text{PGF}_{2\alpha}$ serum. $\times 90$.

Fig. 2 Hippocampal sections from a normal gerbil. A: $\times 66$. B, C: Neither hippocampal pyramidal neurons in the CA1 sector (B) nor granule cells in the dentate gyrus (C) are stained with anti-PGF_{2 α} serum. No blood vessels are stained. The nuclei are counterstained with hematoxylin. $\times 400$.

Fig. 3 Hippocampal sections from a gerbil reperfused for 5 minutes after bilateral carotid occlusion. A: $\times 66$. B, C: Both pyramidal neurons in the CA1 sector (B) and some granule cells in the dentate gyrus (C) are strongly stained with anti-PGF_{2 α} serum. $\times 400$.

the corpus callosum, were stained, but not as intensely as the neurons (Fig. 5C).

PGF_{2 α} staining was not observed with normal rabbit serum. Staining with PGF_{2 α} -absorbed anti-PGF_{2 α} serum resulted in a significant decrease in staining intensity in sections from reperfused gerbils, although slight staining persisted in the vessel walls and hippocampal pyramidal neurons (Fig. 4B). Staining for PGF_{2 α} in reperfused animals was substantially diminished by pretreatment with 10 mg/kg indomethacin given i.p. (Fig. 4C).

Fig. 4 A, B: Hippocampal specimens from a reperfused gerbil stained with anti-PGF_{2 α} serum (A) and anti-PGF_{2 α} serum absorbed by PGF_{2 α} (B). A, $\times 40$; B, $\times 33$. C: Hippocampal section from a different reperfused gerbil pretreated with indomethacin (10 mg/kg, i.p.) stained with anti-PGF_{2 α} serum. Staining is substantially diminished by pretreatment with indomethacin. $\times 33$.

Similar results were obtained with anti-PGF_{2 α} sera from East-Acres Biologicals and Bio-Yeda Ltd.

Discussion

In this study we used immunohistochemical tech-

Fig. 5 Brain sections from a recirculated gerbil, showing marked $\text{PGF}_{2\alpha}$ staining of pyramidal neurons in the CA1 sector (A), of neurons and glial cells in the parietal cortex (B), of glial cells in the corpus callosum (C), and of intraparenchymal vessels (D). The arrow indicates a nucleus. A, $\times 250$; B, $\times 250$; C, $\times 180$; D, $\times 120$.

niques to determine the sites of $\text{PGF}_{2\alpha}$ production following reperfusion of ischemic gerbil brains. Although the anti- $\text{PGF}_{2\alpha}$ serum we used cross-reacts with other PGs, as described above, it reacted mainly with $\text{PGF}_{2\alpha}$ in these experiments, because PGD_2 , PGE_2 , and $\text{PGF}_{2\alpha}$ are the major PGs in the gerbil brain.^{3,7,13} Negative staining with normal rabbit serum and the results of the absorption studies proved anti- $\text{PGF}_{2\alpha}$ serum to be specific to the $\text{PGF}_{2\alpha}$ antigen in the gerbil brain. Pretreatment with indomethacin also confirmed the specificity of the anti- $\text{PGF}_{2\alpha}$ serum to cyclo-oxygenase products.

In the control animals, the vessel walls and intravascular components were faintly stained with anti- $\text{PGF}_{2\alpha}$ serum, with less intense or no staining in neurons and glial cells. On the other hand, after 5 minutes of reperfusion, neurons, glial cells, and blood vessels were markedly stained with anti- $\text{PGF}_{2\alpha}$ serum. These results are consistent with our previous finding, also obtained by indirect immunofluorescence, that staining with anti- $\text{PGF}_{2\alpha}$ serum was markedly increased in the cytoplasm of neurons, oligodendrocytes, and blood vessels after reperfusion in globally ischemic rat brains.¹⁷ Gaudet

*et al.*⁷) reported a rapid increase in PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$, thromboxane B_2 , and 6-keto- $\text{PGF}_{1\alpha}$ upon recirculation after 5 minutes of forebrain ischemia in Mongolian gerbils. Our results corroborate their observations.

Cerebral tissues, incubated *in vitro*, were shown to produce several arachidonate metabolites, including cyclo-oxygenase products, such as $\text{PGF}_{2\alpha}$ and PGE_2 .^{1,25,32} The individual brain tissues and cells that synthesize these PGs, however, were not clearly identified in those investigations. Although some evidence suggests that arachidonic acid metabolites are formed primarily in cerebral vessels rather than in neuronal or glial elements,⁸⁻¹⁰ there are other data that support our results. Seregi *et al.*²²) reported that cultured astrocytes produced large amounts of PGD_2 , PGE_2 , and $\text{PGF}_{2\alpha}$. Fontana *et al.*⁵) also demonstrated that PGE_2 was released from cultured astrocytes and C_6 glioma cells.

Cyclo-oxygenase activity has been observed in homogenates of certain neuroblastoma cell line.^{2,23} Crockard *et al.*⁴) investigated regional levels of brain $\text{PGF}_{2\alpha}$ and PGE_2 in gerbils subjected to bilateral carotid artery occlusion and found an increase in

PGF_{2α} in all areas within 1 hour of occlusion. Using perfusion with an indomethacin solution, these investigators ruled out the vascular component as a contaminating source and concluded that the bulk of the PGs were produced in ischemic brain tissues. These results, together with ours, suggest that the increased production of brain PGF_{2α} during reperfusion is due to increased PG synthesis not only in blood vessels but also in neurons and glial cells.

Recent studies have suggested that PGs may be involved, *via* vasoactive functions, in pathophysiological events following brain ischemia.^{3,6,12,19,26} PGF_{2α} is a vasoconstrictor and PGE₂ can cause either vasoconstriction or vasodilation, depending on the species and the type of blood vessel.^{15,19,30,31} Therefore, it can be postulated that PGs affect cerebral blood flow. Arachidonic acid products, such as leukotrienes and, to a lesser extent, PGE₂, have been shown to influence vascular permeability.²⁶ Kimura *et al.*¹⁴ reported that PGD₂, PGE₂, and PGF_{2α} potentiate the postsynaptic actions of inhibitory and excitatory amino acids in cerebellar Purkinje cell dendrites. PGs, especially PGE₂, have also been reported to inhibit catecholamine release from slices of rat brain cortex²⁰ and from synaptosomes isolated from rat hippocampus.²⁹ These observations indicate that excessive PG production may play a role in the pathophysiological consequences of brain ischemia through modulation of blood flow, vascular permeability, and neurotransmission. Recently, we demonstrated that indomethacin, a cyclooxygenase inhibitor, ameliorated delayed neuronal death in the hippocampal CA1 sector in the same gerbil model.²¹ This may indicate an important role of PGs in the development of delayed neuronal injury, although in the present study the increase in hippocampal PGF_{2α} was not restricted to the CA1 sector. The precise mechanism of indomethacin's effect on delayed neuronal death is still unclear.

Clarification of the role of PGs in neural function requires identification of the sites of binding proteins specific for PGs as well as localization of each PG under normal and pathological conditions. Binding proteins specific for PGD₂, PGE₂, and PGF_{2α} have been found in synaptic membranes and synaptosomes of mammalian animal brains^{16,24} and post-mortem human brains.²⁷ Moreover, PGD₂, PGE₂, and PGF_{2α} binding sites have been localized in monkey brains by the use of autoradiology.²⁸ These binding proteins may be responsible for the initial triggering of PG activity in the brain. These observations suggest the increase in the amount of PGs in a certain region may be related to some functional changes in that region.

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