The Effect of Incomplete Cerebral Ischemia on Prostaglandin Levels in Rat Brain

E. SHOHAM, PH.D., J. ROSENTHAL, M.SC., AND S. LAVY, M.D.*

SUMMARY Rats were subjected to severe incomplete cerebral ischemia followed by recirculation. The levels of several of the cyclooxygenase products of arachidonic acid were measured at 5 and 15 minutes of ischemia and at 30 minutes of recirculation following 15 minutes of ischemia. 

PGF₂α accumulated during the first 5 min. of ischemia and its level declined at 15 min. and returned to control level at 30 min. of recirculation. TXB₂, on the other hand, increased during the whole time course of the experiment and at the end of the post ischemic period its level was 5 times higher than control. Treatment of the animals with indomethacin (4 mg/Kg, i.v.) prior to ischemia reduced the levels of these products without altering the pattern of their changes. During the ischemic period the EEG was isoelectric and the mean recovery time of electrical cortical activity after 15 min. of ischemia was 10.4 ± 3.5 min. in the control rats. The rats which received indomethacin recovered faster (4.3 ± 0.9 min) and were more resistant to the induction of ischemia. We suggest that the reversibility of cortical activity may be correlated to the accumulation of TXB₂ during ischemia and recirculation, and inhibition of its synthesis might improve the post-ischemic reflow.

CEREBRAL ISCHEMIA leads, among other biochemical changes, to the decomposition of membrane-bound phospholipids and a release of free fatty-acids, likely due to the activation of endogenous phospholipases. This breakdown of structural lipids may interrupt membrane function and bring about the accumulation of free fatty acids. The increase in the level of fatty acids, in particular of arachidonic acid, the precursor of prostaglandins, leads to changes in prostaglandin level. An animal model for reversible incomplete ischemia has been developed and studied by Nordstrom et al. and Nordstrom and Siesjo. This (physiologically well controlled) model is based on the occlusion of both carotid arteries of the rat, combined with arterial hypotension. Removal of the clamps from the arteries followed by blood infusion restores the blood supply to the brain. During ischemia the cerebral blood flow in cortical tissue is reduced to less than 10% of normal, the tissue is depleted of ATP following 2-3 minutes of ischemia, and the tissues pool of adenine nucleotides falls rapidly, thus reducing the energy production of the tissue. Recently, Rehncrona et al reported a rapid increase in cerebral cortical content of free fatty acids after 5 minutes of ischemia, in the same model. The accumulation of these FFA is reversed during 30 minutes of recirculation. They also observed a marked (3-fold) increase in the relative content of arachidonic acid.

During the recirculation period following ischemia the neurophysiological and metabolic functions might be restored if no irreversible cell damage occurred. A prerequisite for recovery is an adequate perfusion, whereas immediate or delayed perfusion defects might be the cause for irreversibility of brain function. Hossmann reviewed various factors that may contribute to delayed hypoperfusion thus increasing the primary ischemic region. Among these factors are blood coagulation and vascular spasm.

The maintenance of normal tissue perfusion depends on a balanced interaction of two prostaglandins which have opposite effects at the blood-endothelial interface, namely thromboxane A₂ (TXA₂) and prostaglandin I₃ (PGL₃). Both prostaglandins have the same precursor, cyclic-endoperoxide (PGH₂), but while TXA₂ is a potent platelet aggregator and vasoconstrictor, PGL₃ inhibits platelet aggregation and is a vasodilator. Thus, any interruption in the balanced production of these compounds which might result in an increase of TXA₂ could diminish local blood flow. Hallenbeck and Furlow have shown that dogs exposed to complete ischemia had low post ischemic blood flow with focal zones of greatly impaired reperfusion. A significant increase in the blood flow during the post ischemic period was observed in animals receiving either indomethacin prior to ischemia or a combination of indomethacin and PGI₂ after ischemia. Gaudet and Levine have demonstrated that gerbils pre-
treated with indomethacin are more active after total cerebral ischemia than those without treatment. Thus, the involvement of the prostaglandin system in the outcome of an ischemic event is strongly suggested.

In the present studies we have investigated the changes in the levels of prostaglandin in the cortex of rats after incomplete ischemia and recirculation. Since TXA$_2$ and PGI$_2$ have very short half lives (30 seconds and 3 minutes at 37°, pH 7.5, respectively), their stable metabolites, TXB$_2$ and 6-keto-PGF$_{1a}$ were determined. These metabolites are the products of non enzymatic processes and their levels reflect the levels of the active but unstable compounds. PGE$_2$ is stable enough to be assayed by itself. We suggest a correlation between the effect of reducing prostaglandin synthesis by indomethacin and the improvement of cortical activity as expressed by increased rate of recovery of the EEG.

Materials and Methods
Male albino rats, weighing about 300 g were used for the experiments. The animals were tracheostomized and cannulated in the femoral vein and arteries, under 3% halothan in 70% nitrous oxide and 30% oxygen. The halothan was then withdrawn and the experiments were conducted under light nitrous oxide anesthesia. EEG was recorded by means of gold plated copper screws inserted into the skull bone in the frontotemporal regions. The reversible incomplete ischemia was produced according to Nordstrom and Siesjo and Nordstrom et al. The carotid arteries were occluded by clamps and the blood pressure was reduced by bleeding the femoral artery until the mean arterial pressure (MABP) reached 50 mm Hg.

At this stage the EEG disappeared and that time marked the onset of ischemia. During the ischemic period (5 or 15 minutes) the MABP was kept constant at 50 mm Hg by manually infusing or extracting blood via the femoral vein or artery respectively. To start recirculation, the clamps were removed from the carotid arteries and within 1 minute blood was infused until normal MABP was reached. The EEG was recorded during the whole experiment and served as a tool for evaluating the cortical activity. The experiment was terminated by freezing the brain in situ according to Ponten et al. and subsequently, the brains were chiseled out and kept at −70° until assay.

Groups of animals were studied at 5 and 15 minutes of ischemia, at 15 minutes of ischemia followed by 30 minutes of recirculation, controls consisted of animals subjected to sham operation. In one of these control groups and carotid arteries were exposed but not occluded, and the MABP was kept normal. In the other, the MABP was reduced to 50 mm Hg with no occlusion of the carotid arteries.

For measurement of the effect of indomethacin on prostaglandin accumulation and on recovery of the animal during the post-ischemic period, 4 mg/Kg of the drug (Teva Pharmaceutical Industries Ltd.) was dissolved in saline, the pH was adjusted to 8.0 with NaOH, and infused intravenously about 30 minutes prior to the onset of ischemia. In one group of animals, indomethacin was administered at termination of ischemia, when circulation was restored. The dose of the drug was chosen so that the cyclooxygenase enzyme should be inhibited rather than non specific effects which occur at higher doses. One group of animals received flufenamic acid at a dose of 10 mg/Kg. The drug was dissolved in NaOH pH 8.0 and diluted in saline before injection.

Assay of Prostaglandin
The concentrations of prostaglandins in cortical tissue were determined by RIA using a specific rat antibody serum. These assays were performed according to Weidenfeld et al., for PGE$_2$, and Ligumski et al. for TXB$_2$, and 6-keto-PGF$_{1a}$, and their same batch of antibody preparations was used. The tissue was homogenized in Tris-EDTA buffer, (0.05M Tris + NaCl and 0.2M EDTA at 9:1 ratios, pH 7.0). The homogenates were washed twice with 2 volumes of ether, and the aqueous phase was stored frozen until assay. RIA was carried out in 0.01M potassium phosphate buffer pH 7.4 containing 0.1% sodium azide and 0.1% bovine serum albumin, and the reference preparations used were PGE$_2$ (Radiochemical Centre, Amersham, England), 6-keto-PGF$_{1a}$ and TXB$_2$ (New England Nuclear Sample, standard tracer and antiserum were incubated overnight at 4°C. Separation of bound and free fractions was achieved with charcoal (Norit GSX Activated). Radioactivity was counted in a liquid scintillation counter.

The antibody for 6-keto-PGF$_{1a}$ was kindly donated by Dr. A. Eldor from Hadassah. The antibodies for PGE$_2$, TXB$_2$, were raised by Dr. F. Cohen from the Weizmann Institute. Data was analyzed by two tailed t test.

Results
Prior to each experiment mean arterial blood pressure and blood gases were measured and these are described in table 1, for the control, and in table 2 for the treated animals. All the variables listed are within the normal range and there were no significant differences between the various groups. Rectal temperature was maintained at about 37°C. These measurements ensured us that in all animals, untreated as well as treated with indomethacin, ischemia was induced under comparable conditions.

The effect of incomplete ischemia on concentrations of PGE$_2$, 6-keto-PGF$_{1a}$, and TXB$_2$ in the rat cortex is shown in figure 1. Groups 3–5 represent respectively these levels at 5 and 15 minutes of ischemia and at 30 minutes of reperfusion following 15 minutes of ischemia. These levels are compared with the two control groups that were sham operated with normal (control group 1) and reduced MABP (control groups 2), as described in Materials and Methods.

The concentration of PGE$_2$ (fig. 1a) increased about twofold after 5 minutes of ischemia, declined after 15 minutes, and following 30 minutes of recirculation it returned to control levels. The concentrations of PGE$_2$
in the two control groups do not differ, indicating that the increase that occurs during the ischemia was due to ischemia and not to bleeding alone.

The pattern of changes in the levels of 6-keto-PGF$_{1\alpha}$ is depicted in figure 1b. An increase during 5 and 15 minutes of ischemia (columns 3 & 4 of figure 1b) is followed by a decline to the normal levels after 30 minutes of recirculation (column 5). However, the level of 6-keto-PGF$_{1\alpha}$ in control group 2, where MABP was reduced to 50 mm Hg by hemorrhage, was not significantly different from its level after ischemia. Thus, in the present model it is impossible to conclude whether the increase of 6-keto-PGF$_{1\alpha}$, as a result of the clooxygenase step, one group of animals was given flufenamic acid prior to the ischemia, and the levels of the PG's were determined after the recirculation period. The levels of these products measured after recirculation were not significantly different from their counterparts in which the drug was given prior to ischemia.

In order to establish the fact that the differences observed in prostaglandin levels, as well as the recovery from ischemia, were due to inhibition at the cyclooxygenase step, one group of animals was given flufenamic acid prior to the ischemia, and the levels of the PG's were determined after the recirculation period. PGE$_2$ and TXB$_2$ levels showed the same pattern as untreated rats, but their levels were reduced (20–60%), whereas 6-keto-PGF$_{1\alpha}$ was higher in the control group and after 5 minutes of ischemia, and lower after 15 minutes of ischemia and after the recirculation period.

The EEG. The EEG was isoelectric for the whole period of ischemia and the first period of the recirculation period. The levels of these products measured after recirculation were not significantly different from their counterparts in which the drug was given prior to ischemia.

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**Table 1. MABP, Blood Gas Values and pH Measurements Prior to Ischemia (mean ± S.E.): Untreated Animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>n = no. of animals</th>
<th>MABP mmHg</th>
<th>pO$_2$ mmHg</th>
<th>pCO$_2$ mmHg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control Normal MABP</td>
<td>8</td>
<td>135 ± 5</td>
<td>83.3 ± 4.3</td>
<td>36.6 ± 1.3</td>
<td>7.39 ± 0.02</td>
</tr>
<tr>
<td>2. Control 50 mmHg MABP</td>
<td>6</td>
<td>124 ± 3</td>
<td>84.0 ± 5.3</td>
<td>33.6 ± 0.5</td>
<td>7.38 ± 0.05</td>
</tr>
<tr>
<td>3. Ischemia 5'</td>
<td>6</td>
<td>122 ± 6</td>
<td>81.6 ± 6.0</td>
<td>38.9 ± 4.2</td>
<td>7.36 ± 0.05</td>
</tr>
<tr>
<td>4. Ischemia 15'</td>
<td>6</td>
<td>125 ± 6</td>
<td>79.2 ± 8.2</td>
<td>36.2 ± 1.2</td>
<td>7.39 ± 0.02</td>
</tr>
<tr>
<td>5. 15' Ischemia + 30' recirculation</td>
<td>12</td>
<td>129 ± 3</td>
<td>94 ± 4</td>
<td>37.8 ± 1.2</td>
<td>7.43 ± 0.02</td>
</tr>
</tbody>
</table>

**Table 2. MABP, Blood Gas Values and pH Measurements Prior to Ischemia (mean ± S.E.): Treated Animals — 4 mg/Kg Indomethacin or 10 mg/Kg Flufenamic Acid**

<table>
<thead>
<tr>
<th>Group</th>
<th>n = no. of animals</th>
<th>MABP mmHg</th>
<th>pO$_2$ mmHg</th>
<th>pCO$_2$ mmHg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. Control</td>
<td>5</td>
<td>131 ± 6</td>
<td>107.1 ± 1.9</td>
<td>36.0 ± 1.7</td>
<td>7.39 ± 0.01</td>
</tr>
<tr>
<td>7. Ischemia 5'</td>
<td>5</td>
<td>124 ± 1</td>
<td>93.8 ± 7.3</td>
<td>34.3 ± 1.3</td>
<td>7.42 ± 0.02</td>
</tr>
<tr>
<td>8. Ischemia 15'</td>
<td>7</td>
<td>120 ± 6</td>
<td>84.5 ± 14.5</td>
<td>30.4 ± 0.8</td>
<td>7.43 ± 0.02</td>
</tr>
<tr>
<td>9. Ischemia 15' + 30' recirculation</td>
<td>9</td>
<td>119 ± 2</td>
<td>88.0 ± 4.6</td>
<td>37.7 ± 1.3</td>
<td>7.37 ± 0.02</td>
</tr>
<tr>
<td>10. Flufenamic acid, ischemia 15' + 30' recirculation</td>
<td>5</td>
<td>138 ± 1</td>
<td>91.7 ± 6.0</td>
<td>38.6 ± 1.1</td>
<td>7.38 ± 0.03</td>
</tr>
</tbody>
</table>
EFFECT OF INCOMPLETE CEREBRAL ISCHEMIA ON PROSTAGLANDIN

A B C

FIGURE 1. Concentrations (pg/mg tissue) of PGE$_2$ (A), 6-keto-PGF$_{1α}$ (B) and TXB$_2$ (C) in the cortex of rats subjected to ischemia and recirculation. Experimental groups 1–5, as described in table 1. ***p ≤ 0.0005 (two tailed t-test). **p ≤ 0.02. *p ≤ 0.05.

the animal. Since the animals were anesthetized no behavioural parameters could be used for the evaluation of the state of the animal. The depth of anesthesia during the experiment was the same in all animals since they were constantly ventilated by the same mixture of gases, as described above. In 31% of animals (5 out of 16), no sign of recovery of the EEG was noticed during the 30 minutes of recirculation. The average recovery time for the remainder of the animals was 10.4 ± 3.5 minutes.

In the group of animals treated with indomethacin prior to ischemia the mean recovery time was 4.3 ± 0.9 minutes, and only one animal (out of 11) did not recover at all. Three animals (27%) had residual EEG activity during the ‘‘Ischemic’’ period, in other words in these animals we could not induce ischemia under the usual conditions.

In the group of animals which were treated with indomethacin after the ischemia, the mean recovery time was 7.4 ± 1.3 minutes.

The mean recovery time of the EEG of the animals pretreated with flufenamic acid was 5.6 ± 1.6 minutes, one animal (16%) did not recover during 30 minutes of recirculation and one animal had a residual activity during the ischemic period.

Discussion

The present study was carried out in order to explore the involvement of the prostaglandin system in the biochemical changes that occur during and after brain ischemia. The sampling points were separated by time so that transient changes that might occur immediately after recirculation could have been overlooked. The time points were chosen so as to get steady state values of the prostaglandins. We found that PGE$_2$ levels increased within 5 minutes of ischemia, declined at 15 minutes of ischemia and returned to normal values at 30 minutes of recirculation. Thus although some changes occur in the levels of PGE$_2$, these changes were reversible with the time course of the experiment. The pattern of changes is parallel to the pattern of arachidonic acid accumulation under the same conditions. Indomethacin treatment reduced the levels of PGE$_2$ by about 20% at all the time points measured, without affecting the trend of changes.

As mentioned above, the changes in 6-keto-PGF$_{1α}$ levels, were similar to those of PGE$_2$, but they are not relevant to this model since the same increase that occurs at ischemia might be due to the bleeding. This increase could be attributed to an autoregulatory response to the reduction of blood pressure.

The major effect of the ischemia was found to be on TXB$_2$, which remained high (4–5 times of the control levels) even after 30 minutes of recirculation. The balanced interaction between the production of TXA$_2$ and PGI$_2$ was disturbed as a result of the ischemia in such a way that TXA$_2$ accumulated more than PGI$_2$. This disproportionate production of TXA$_2$ could diminish

A B C

FIGURE 2. Concentrations of PGE$_2$ (A), 6-keto-PGF$_{1α}$ (B) and TXB$_2$ (C) in the cortex of rats treated with 4 mg/kg indomethacin (i.v.) 30 mins. prior to ischemia. Experimental groups 6–9, as described in table 2. ***p ≤ 0.0005 (two tailed t-test). **p ≤ 0.02. *p ≤ 0.05.
local blood flow and it was shown by Hallenbeck and Furlow that post ischemic reperfusion was improved after administration of indomethacin prior to ischemia. Our results suggest that the particular prostaglandin involved with the impairment of post-ischemic brain reperfusion might be TXA₂. We have shown that reduction of TXB₂ levels by indomethacin pretreatment improved the recovery of the cortical activity as expressed by the shorter “recovery time” of the EEG. Although we did not measure cerebral blood flow in our experiments, the results are compatible with a prevention by indomethacin of the impairment of post ischemic reflow. Our results also confirm Hallenbeck and Furlow's that the efficacy of the indomethacin is higher when administered prior to, rather than following ischemia. The different pattern of changes in the levels of PGE₂, PGF₁, and TXA₂, as a result of ischemia might be due to a different sensitivity of the enzymes isomerase, prostacyclin-synthetase and thromboxane synthetase, to the ischemic insult.

There is evidence that the cyclooxygenase in the platelets is more sensitive to inhibition by aspirin than is the enzyme that synthesizes PGF₁, in the vessel wall. It seems from our results that the same is true for inhibition by indomethacin. While PGE₂ levels were slightly reduced after indomethacin, TXB₂ was reduced dramatically, whereas PGF₁ was hardly affected by that drug. Thus the differential effects on the products of the endoperoxide result from different sensitivity of the relevant enzymes either to the pathologic condition or to the drug.

Flufenamic acid, administered prior to ischemia, had a similar effect on the PG’s levels and on the outcome of ischemia as indomethacin. Since this drug is known to inhibit the same step in the biosynthesis of TXA₂ as does the indomethacin, namely the cyclooxygenase, it is probable that inhibition indeed occurred at this particular stage.

The effect of incomplete ischemia on the levels of prostaglandins in the rat cortex agrees well with the increase in free fatty acid pools which was observed in the same and other models. The increased availability of arachidonic acid due to ischemia together with the residual oxygen supply which persists during that type of ischemia may explain the increase in prostaglandin contents during the ischemic period. These results do not agree with the findings of Gaudet et al. who reported an increase in prostaglandins only at the reperfusion period, while during ischemia no change was observed. This discrepancy can be explained by the different availability of oxygen in the two models. In Gaudet’s experimental model (bilateral common carotid occlusion) the tissue is completely depleted of oxygen, thus the arachidonic acid which accumulates cannot be metabolized. In the present model, cortical blood flow is about 5–10% of its normal value and this supply might be sufficient to metabolize some of the arachidonic acid which accumulates in the tissue.

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We are particularly grateful to Prof. B. K. Siesjo from the Laboratory of Experimental Brain Research, University of Lund, Sweden, in whose laboratory E. S. studied the technique employed in this work.

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Response of Local Blood Flow in the Caudate Nucleus of the Cat to Intraventricular Administration of Histamine

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SUMMARY The effect of intraventricular histamine on blood flow in the caudate nucleus of the cat was studied by means of the hydrogen clearance technique. Bilateral ventriculo-cisternal perfusion was installed. After a control period during which both lateral ventricles were perfused with mock CSF with the same composition, the drug under study was added to one side (experimental side) while the other side was perfused further with the control mock CSF (control side).

At each point in time, blood flow at the experimental side was compared to that at the control side. Histamine (10^{-3} M) caused a severe vasodilatation and this effect was completely antagonised by the H_3-receptor blocker cimetidine (10^{-2} M). Cimetidine had no vasoactive effects of itself in the concentration used. The H_3-receptor agonist Dimaprit (10^{-3} M) had a vasodilator effect although less important than histamine.

Indirect evidence was gained that H_3-receptors are not active in the vascular bed under study.

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HISTAMINE is a potent vasoactive substance that is present in most tissues in variable concentrations. Direct local application of the substance on superficial brain arteries has shown dilatory effects in cats\cite{1,2,3} although no effect was reported in mice.\cite{4,5,6} Pial arteries in the cat thus appear to dilate in a dose dependent manner upon perivascular application of histamine and this vasodilatation is mediated by H_3-receptors.\cite{2,3,5}

In the present experiments the effect of histamine on the local blood flow in the deeper parenchyma of the cat brain was investigated.

**Materials and Methods**

Experiments were carried out on anesthetized (pentobarbital 30 mg/kg), paralysed (gallamine 10 mg/kg) and artificially ventilated cats (30% O_2, 70% N_2) weighing approximately 3 kg. Appropriate anesthesia and relaxation was maintained by additional doses of pentobarbital (5 mg/kg) and gallamine (3 mg/kg) every hour. The animals were placed in a stereotaxic apparatus and a bilateral ventriculocisternal perfusion (VCP) was installed in a similar way as described in a previous communication of our laboratory.\cite{6} Two inlet-cannulae, one on each side, were lowered in the lateral cerebral ventricles with a microdrive system. Through these cannulae, artificial cerebrospinal fluid was administered at a rate of 0.123 ml/min (Harvard Infusion-withdrawal pump model 901). An outlet cannula was placed in the suboccipital cistern. The perfusion pressure was continuously monitored at both sides. The composition of the mock CSF was as follows (mmol/l): NaCl 138; KCl 3.3; NaHCO_3 25.0; NaHPO_4; H_2O 0.5; MgCl_2;H_2O 1.2; CaCl_2 1.25; glucose. H_2O 3.1.

Substances under study were added to the mock CSF. Osmolality and bicarbonate concentration were carefully checked and if necessary adjusted to 320 mOsm/kg and 25 mmol/l respectively. Blood gases were controlled and the animals were kept in steady state normocapnia (paco_2 30-40 mm Hg) by adjustment of ventilation. Blood pressure was monitored during the entire experiment; in the experiments reported mean blood pressure was at least 100 mm Hg.

**CBF measurement**

Blood flow was measured simultaneously in both caudate nuclei with the hydrogen clearance method.\cite{2,10} Two hydrogen sensitive electrodes (glass insulated platinized platinum iridium wire \( \varnothing 0.35 \text{ mm} \) — one on each side — were stereotaxically placed in the head of each caudate nucleus (coordinates A_{17}; L_{4}; H_{2}) according to the stereotaxic atlas of Snider and Niemer.\cite{11} The animals were saturated with hydrogen, by adding 10% hydrogen gas to the inspired air. Hydrogen ad-
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