Prostaglandin Release From Isolated Rabbit Cerebral Cortex Micro-Vessels — Comparison of 6-Keto PGF<sub>1α</sub> and PGE<sub>2</sub> Release From Micro-Vessels Incubated in 100% O<sub>2</sub>, Room Air and 95% N<sub>2</sub>:5% CO<sub>2</sub>

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SUMMARY Prostaglandin release from microvessels isolated from the rabbit cerebral cortex was determined under three different atmospheric conditions: 100% O<sub>2</sub> ("O<sub>2</sub>") room air, and 95% N<sub>2</sub>:5% CO<sub>2</sub> ("N<sub>2</sub>-CO<sub>2</sub>"). Initial studies with homogenates prepared from rabbit cerebral microvessels (RCMV) indicated two pathways of enzymatic PGH<sub>2</sub> transformation, namely PGI<sub>2</sub> synthase and GSH-dependent PGH-PGE isomerase. We measured the release of the principal products of these pathways, 6-keto PGF<sub>1α</sub> and PGE<sub>2</sub> from freshly prepared RCMV. The release of 6-keto PGF<sub>1α</sub> exceeded that of PGE<sub>2</sub> in all three protocols. RCMV incubated in "N<sub>2</sub>-CO<sub>2</sub>" exhibited a reduction in the release of 6-keto PGF<sub>1α</sub> compared to room air or "O<sub>2</sub>". Incubated RCMV, evident at 30-60 min of incubation. No significant differences in the release of PGE<sub>2</sub> were observed among the three incubation protocols. In all three incubation protocols the ratio of 6-keto PGF<sub>1α</sub> to PGE<sub>2</sub> did not differ during the initial 10 minutes of each incubation. After 30 to 60 min of incubation, this ratio did not change from the "O<sub>2</sub>" or room air treated RCMV, but decreased significantly for the "N<sub>2</sub>-CO<sub>2</sub>" treated group. To determine the reversibility of the apparent "N<sub>2</sub>-CO<sub>2</sub>" induced decline in 6-keto PGF<sub>1α</sub> release, microvessels were removed from the nitrogen atmosphere and incubated in room air. Release was measured during the initial 10 min following reintroduction to room air and was compared to room air pretreated controls treated in an identical manner. Complete recovery of 6-keto PGF<sub>1α</sub> production was observed, and an enhanced ratio of 6-keto PGF<sub>1α</sub> to PGE<sub>2</sub> observed in the "N<sub>2</sub>-CO<sub>2</sub>" treated RCMV. In view of the opposing actions of PGI<sub>2</sub> (vasodilator) and PGE<sub>2</sub> (mild vasoconstrictor) on cerebrovascular tone, this study suggests that release of prostaglandins by the microcirculation may participate in the cerebrovascular response to ischemia. In addition, this study suggests that the release of 6-keto PGF<sub>1α</sub> and PGE<sub>2</sub> by cerebral microvessels may be regulated independently.

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acid and other free fatty acids in the brain during or following ischemia, hypoglycemia and epileptic episodes. In ischemia, the depletion of brain oxygen levels may reduce the metabolism of arachidonic acid to PGs and related substances. However, a burst of PG synthesis has been observed following post-ischemic recirculation. Ellis et al. found that hypoxia was not associated with any significant alteration in brain-tissue levels of PGE₂, PGF₂α or 6-keto PGF₁α in the cat, although there was a trend for decrease in PGE₂ levels during hypoxia that neared statistical significance. In the same study, arterial hypercapnia was associated with a substantial decrease in tissue levels of PGE₂ and PGF₂α, with a tendency toward a decline in 6-keto PGF₁α, suggesting that more severe hypoxia, perhaps combined with hypercapnia, may be required to bring about increased arachidonate release followed by subsequent increases in PG formation. In the study by Ellis et al., levels of PGE₂ and PGF₂α were five times higher than 6-keto PGF₁α. The levels of 6-keto PGF₁α did not alter significantly during hypoxia or hypercapnia. It has been suggested that 6-keto PGF₁α formation in the brain is vascular in origin, although the vasculature constitutes a relatively small percentage of the cerebral cortex. It is possible, therefore, that altered release of PGs (6-keto PGF₁α) might be difficult to detect in whole cerebral preparations.

In the present study, we report on our studies investigating the effects of various atmospheric conditions on the release of prostaglandins from isolated microvessels prepared from the rabbit cerebral cortex. Microvessels were incubated in three different atmospheric conditions (100% O₂, "N₂-CO₂") at 37°C. Release of 6-keto PGF₁α and PGE₂ from the microvessels was measured at various time intervals (1 min to 60 min) of incubation under these conditions. The results from these studies indicated that the synthesis and release of 6-keto PGF₁α in isolated microvessels can be significantly altered with variations in the atmospheric conditions. Compared with microvessels incubated in room air, release of 6-keto PGF₁α was depressed at later time points during the incubation with "N₂-CO₂", but did not differ from microvessels incubated with "O₂".

Methods

Cerebral Microvessel Isolation Procedure

Adult male white New Zealand rabbits (1–2 kg) were sacrificed by cervical dislocation (6 in each experiment). The cortex and midbrain were removed quickly and placed into cold PBS on ice. All subsequent procedures were done at 4°C. The cerebellum and midbrain were removed and the remaining cortical mantle was freed of the pial membranes, arachnoid matter and any obvious large vessels. Microvessels were isolated by the method of Selivonchick and Root. The resulting tissue was homogenized in 10 volumes of ice cold phosphate buffered saline (PBS), pH 7.4, using a Dounce homogenizer and 25 up and down strokes. The homogenate was poured over an 88 μm nylon sieve. The retentate was resuspended in ice cold PBS in 50 ml (Sorvall) centrifuge tubes. The suspension was centrifuged at 1500 x g for 10 min, the pellet resuspended in 0.25 M sucrose and layered over a discontinuous gradient, 12 ml each, of 1.0 M and 1.5 M sucrose. This was centrifuged at 58,000 x g for 45 minutes (4°C) in a Beckman ultracentrifuge using a Beckman SW 25.1 rotor. The microvessel pellet was resuspended in 4°C PBS and filtered over a 20 μm sieve. The retentate was thoroughly rinsed with PBS, gently removed from the sieve and used immediately.

In another study, homogenates of microvessels were prepared by mincing with razor blades followed by sonication (Branson sonifier) in 100 mM Tris-HCl buffer, pH 8.1. Homogenates were used immediately for PGH₂ incubations.

Incubation Protocol

The isolated microvessels were divided into three groups and weighed. Each group was placed into nylon sieve (20 μm) baskets, which were subsequently put into individual 25 ml erlenmeyer flasks, stopped with rubber stoppers. Each stopper was fitted with three 23 gauge needles. Two needles were used for the inflow and outflow of gas. The third needle was fitted with a tuberculin syringe for sample removal and a three-way stopcock, enabling buffer removal without disturbance of the atmospheric conditions or the microvessels. Each flask contained 6 ml of PBS at 37°C and had been pre-equilibrated with one of room air II. 100% O₂ or III. 95% N₂:5% CO₂ for 30 min prior to addition of the microvessels. The microvessel basket was rapidly inserted at time 0, the stoppers immediately replaced, and incubated in a 37°C shaking water bath with constant flow of the appropriate gas to the flasks to maintain initial conditions. A 500 μl aliquot was removed at times ranging from 1–60 minutes. In trials 10–14, following the 60 minute time point, groups I and III were transferred to another 25 ml flask containing 6 ml of fresh PBS (37°C) and incubated in room air only. A 500 μl aliquot was taken at 1, 5 and 10 minutes.

Each aliquot was frozen and subsequently assayed for 6-keto PGF₁α and PGE₂ by radioimmunoassay.

[^14C] PGH₂ Metabolism

Homogenates prepared from rabbit cerebral microvessels were incubated with [1-^14C]PGH₂ (prepared from [1-^14C] arachidonic acid (52 mCi/mmol) following the protocols outlined in earlier publications. Incubations were performed in the presence or absence of 2 mM glutathione, and boiled homogenates and rabbit serum albumin were controls. Products were extracted with 3.5 volumes of ethyl acetate: methanol:0.2M citric acid (15:2:1 v/v), the organic phase applied to silica gel GHL thin-layer chromatography (TLC) plates along with authentic standards (kindly provided by Upjohn Co., Kalamazoo, MI). Chromatograms were developed in the organic phase of ethyl acetate:hexane, acetic acid:water (56:24:12:60) and quantification achieved by radiochromatogram scanning (Packard 7201) and scraping and counting appropriate zones of the TLC plates.
Radioimmunoassay of PGE₂ and 6-keto PGF₁₀

The concentration of immunoreactive 6-keto PGF₁₀ (the stable hydrolytic product of PGI₂) and immunoreactive PGF₂, were determined in nonextracted samples of PBS that had been incubated with isolated rabbit cerebral microvessels. Antisera was kindly supplied by Dr. Lawrence Levine (Brandeis University, Waltham, MA). The radioimmunoassay procedure was performed as detailed by Granström and Kindahl. Antisera was kindly supplied by Dr. Lawrence Levine (Brandeis University, Waltham, MA). The radioimmunoassay procedure was performed as detailed by Granström and Kindahl. The detection limit of both the 6-keto PGF₁₀ and PGF₂, antisera was 10 pg/tube and intra- and interassay variation was routinely less than 10%. Values are expressed as the amount of prostaglandin released/mg wet weight. The anti-6-keto PGF₁₀ antisera demonstrated some cross-reactivity with 6-keto PGI₂ (3.1%), 6,15-diketo PGF₁₀ (1.0%) and PGF₂, (0.3%). The remaining prostanoids tested (13,14-dihydro, 6,15-diketo-PGF₁₀, PGE₂, TxB₂, PGA₂, and arachidonic acid) exhibited cross-reactivity of less than 0.03%. The antisera directed against PGF₂, was not as specific, exhibiting complete cross-reactivity with PGE₂ (150%), and detectable cross-reactivity with PGE₂ (25.6%), PGD₂ (0.4%), PGE₂ (0.3%), 6-keto PGE₂ (10.1%), 15-keto PGE₂ (2.6%). The cross-reactivity with other metabolites of the arachidonic acid cascade (i.e. TxB₂, 6-keto PGF₁₀, 13,14-dihydro, 6,15-diketo PGF₁₀, and arachidonic acid was less than 0.01%.

Statistical Analysis

The time-course of prostaglandin release during incubation in "O₂" or "N₂-CO₂" were compared to room air controls using a two-way analysis of variance. Significance was accepted at p < 0.05. In other experiments, data was compared to room air controls using a Student's t-test (non-paired values).

Results

Metabolism of [1-¹⁴C] PGH₂ by Rabbit Cerebral Microvessel Homogenate

Homogenates (equivalent to 100 µg protein) prepared from rabbit cerebral microvessels were incubated with [1-¹⁴C] PGH₂ (25,000 dpm, 0.2 nmol) in the presence and absence of reduced glutathione (GSH). Control incubations used boiled homogenates or rabbit serum albumin. Two enzymatic pathways of PGH₂ metabolism were observed: PGI₂ synthase, resulting in the formation of 6-keto PGF₁₀, and a PGH-E isomerase, as indicated by the GSH-enhanced formation of PGE₂. Neither activity was detected in boiled homogenate or serum albumin incubations (table 1). The GSH-enhanced PGE₂ forming activity was GSH-specific, and was inhibited by preincubation with 5mM N-ethylmaleimide or 5mM p-hydroxymercuribenzoate (data not shown).

Release of 6-keto PGF₁₀ and PGE₂ by Isolated Rabbit Cerebral Microvessels

Release of 6-keto PGF₁₀ and PGE₂ was measured in 500 µl aliquots removed from the incubations at 1, 5, 10, 30 and 60 minutes. Aliquots were removed carefully with the tuberculin syringe as described in the methods. In all three incubation conditions release of both 6-keto PGF₁₀ and PGE₂ occurred at a similar rate for the initial 10 minutes of incubation. This may be explained, in part, by the disturbance of the equilibrated atmosphere during the insertion of the microvessels. At 30 and 60 min, microvessels incubated in "N₂-CO₂" exhibited a significant reduction in release of 6-keto PGF₁₀ compared to microvessels incubated in room air or "O₂" (fig. 1). The release of PGE did not differ significantly from one incubation condition to another (fig. 2). Microvessels incubated in "O₂" tended to release somewhat larger amounts of 6-keto PGF₁₀ at 30 min, although the differences were not significant. The ratio of 6-keto PGF₁₀ to PGE₂ release at the 15 min time point was significantly lower in "N₂-CO₂" compared to "O₂" and room air incubations (fig. 2).
different time points was also determined (table 2). No significant difference in 6-keto PGF$_{10}$ / PGE$_2$ was observed between microvessels incubated in room air versus "O$_2". However, microvessels incubated in "N$_2$ -CO$_2" demonstrated an initial elevation in the ratio of 6-keto PGF$_{10}/$PGE$_2$, followed by a decline evident at 30 and 60 min. In another series of experiments, microvessels that had been incubated in room air or "N$_2$-CO$_2" for 60 min were removed, placed in a new incubation flask containing PBS and incubated for an additional 10 min. Five hundred µl aliquots were removed at 1, 5, and 10 min. The results from this experiment are shown in figure 3. Upon reintroduction to room air after incubation in "N$_2$-CO$_2" microvessels that had previously demonstrated depressed 6-keto PGF release (fig. 1) exhibited a "rebound" increase in release. Although all of the individual experiments indicated that "N$_2$-CO$_2" pretreated microvessels apparently released greater amounts of 6-keto PGF$_{10}$ than room-air pretreated controls, the variation from one experiment to another was such that "N$_2$-CO$_2" versus room-air pretreated groups did not differ significantly. However, in view of the significant decrease in 6-keto PGF$_{10}$ release after 60 min of incubation in "N$_2$-CO$_2" recovery to room air control levels upon reintroduction into room air indicates that the hypoxia-induced depression in 6-keto PGF$_{10}$ release was reversible. Release of PGE by room air versus "N$_2$-CO$_2" pretreated groups did not differ after reintroduction to room air. The ratio of 6-keto PGF$_{10}/$PGE$_2$ declined from the first to the tenth minute of reintroduction to room air for the "N$_2$-CO$_2" pretreated group. The ratio of 6-keto PGF$_{10}/$PGE$_2$ was significantly higher in the "N$_2$-CO$_2" pretreated microvessels versus the room air control pretreated preparations. Table 3, at the first time point, but did not differ significantly at 5 and 10 min.

**Table 2**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Room air</th>
<th>100% O$_2$</th>
<th>95% N$_2$/5% CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6±2.2</td>
<td>3.6±1.7</td>
<td>8.3±3.0</td>
</tr>
<tr>
<td>5</td>
<td>1.8±0.9</td>
<td>3.4±1.9</td>
<td>7.9±5.7</td>
</tr>
<tr>
<td>10</td>
<td>2.3±0.9</td>
<td>2.8±1.4</td>
<td>3.2±1.6</td>
</tr>
<tr>
<td>30</td>
<td>3.8±2.0</td>
<td>2.8±1.3</td>
<td>2.4±0.9</td>
</tr>
<tr>
<td>60</td>
<td>3.1±1.9</td>
<td>1.8±0.9</td>
<td>1.5±0.7*</td>
</tr>
</tbody>
</table>

*Significantly different (p < 0.05) from ratio at 1 min for that atmospheric condition (S.E.M. n = 14).
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Discussion

Altered blood flow following periods of ischemia is a well-recognized phenomenon with respect to both experimental transient cerebral ischemia. Both "no-reflow phenomenon" and hyperemia have been observed although the mechanisms for these divergent observations remain unclear.

It is conceivable that regional blood flow in the ischemic brain can be altered by the synthesis and release of prostaglandins. These vasoactive substances may derive from the cerebral cortical tissue (i.e. the neural and glial elements) and/or from the cerebral vasculature. Furthermore, it is possible that ischemia may effect neuronal/glial tissue in a different manner than the cerebral vasculature. Release of a vasoconstrictor principle from one element might be balanced by the release of vasodilators from another. Thus, the net effect on regional blood flow following ischemia would be the result of these opposing influences.

The present study addressed the effects of altered atmospheric conditions on prostaglandin release by isolated cerebral microvessels. Incubations with [1-14C] PGH2 indicated that the RCMV exhibited two pathways of PGH2 metabolism, leading to the formation of 6-keto PGF1α and PGE2. Incubation of cerebral microvessels with [14C] PGH2 failed to indicate further metabolism of PGE2 for periods of incubation up to 2 hrs (unpublished observations). Thus, measurement of 6-keto PGF1α and PGE2 release from isolated RCMV is likely to be an accurate measurement of the majority of the prostaglandins released by this tissue. We attempted to measure TXB2 release from the RCMV, but all measurements were lower than the sensitivity of the assay (10-20 pg/tube). Incubations with PGH2 did not indicate the presence of TXA2 synthase activity in the RCMV. We determined 6-keto PGF1α and PGE2 release under various atmospheric conditions to attempt to indicate a possible role for these substances in the processes following an ischemic attack. As previously stated, membrane bound arachidonic acid is rapidly released during hypoxic conditions in the brain and is apparently metabolized by cyclooxygenase only if reflow occurs and tissue oxygen is replenished. A resulting production of certain vasoconstricting prostaglandins (PGs) may cause reduced blood flow. Conversely, vasodilator (i.e. prostacyclin) release may produce hyperemia. Our results indicate that the two major PG products of the cerebral microvasculature are not produced in similar patterns under conditions of varying atmospheric conditions. As expected, the release of 6-keto PGF1α is significantly reduced in the absence of oxygen (fig. 1). However, release of PGE2 did not differ significantly from one experimental condition to another (fig. 2), although at 60 min of incubation, levels of PGE2 release demonstrated somewhat lower (although not significant) levels than corresponding incubation in 100% O2 or room air. The release of 6-keto PGF1α appears to be directly related to the available oxygen supply — RCMV incubated in 100% O2 tended to release somewhat larger amounts of 6-keto PGF1α than room air, and significantly more than 95% N2:5% CO2 incubated RCMV at 30 and 60 minutes. These observations suggest that the release of PGI2 by the cerebral microvasculature of the rabbit may be more sensitive to physiological stimulation, and furthermore, that the release of PGI2 and PGE2 from RCMV are not directly coupled. These RIA results may be somewhat complicated by the observed non-enzymatic isomerization of PGH2 to PGE2, as indicated in table 1. Thus, PGE2 release may not reflect enzymatic production of the prostanooid, but rather the sum of enzymatic and spontaneous isomerization of PGH2.

The results from this study do not support the general theory that hypoxia is a stimulus to enhance prostaglandin release. The results from this study suggest that the rate of release of 6-keto PGF1α may be related to O2 tension, as would be predicted by the O2 requirement of the cyclooxygenase enzyme. Reintroduction of RCMV from a low O2 environment to room air indicated that the 95% N2:5% CO2 induced depression of 6-keto PGF1α release was reversible. One possible explanation for the lack of a significant difference between 100% O2 versus room air incubated preparations might be that O2 concentrations in room air are not rate-limiting.

It is difficult to extrapolate from this reintroduction experiment to the "reflow" in ischemic tissue following hypoxia. However, the net increase in 6-keto PGF1α release in the room air following the 95% N2:5% CO2 incubation is suggestive of an enhanced release of PGI2 following "hypoxia". RCMV incubated in room air for 60 min had released 3.5 ± 0.9 ng/mg wet wt 6-keto PGF1α at 60 min; a 10 min reintroduction to fresh buffer under identical conditions resulted in further release of 0.9 ± 0.4 ng/mg wet wt of 6-keto PGF1α after 10 min. In contrast, microvessels incubated in 95% N2:5% CO2 for 60 min had released 1.3 ± 0.5 ng/mg wet wt 6-keto PGF1α; reintroduction to fresh buffer and room air resulted in release of an additional 1.3 ± 0.3 ng/mg wet wt 6-keto PGF1α.

Throughout all of incubations, the release of 6-keto PGF1α/PGE2 exceeded that of PGE2. This ratio was depressed following 30-60 min incubations in "N2:CO2" compared to the ratio at one minute.

The ratio of 6-keto PGF1α/PGE2 was enhanced when microvessel preparations under hypoxic conditions were transferred to normal room air conditions after the 60 minute time point (table 3), indicating that the stimulation of PG products upon restoration of oxygen appears to favor PGI2. The levels of PGE2 released from the oxygen deprived microvessels did not differ from the room air controls when the preparations were reintroduced to room air.

A known physiological cerebral vasodilator has been implicated in the hyperemia observed during reperfusion following transient brain ischemia. Several investigators report that PGI2 reverses contractions of cerebral vessels and produces enhancement of post-ischemic reperfusion. It has also been revealed that a combination of indomethacin and PGI2 treatment during reperfusion will increase CBF,
possibly due to an imbalance in PG pathways at the endothelial interface.\(^1\)\(^2\) In light of these findings, a high ratio of PGI\(_2\) to PGE\(_2\) production in the cerebral circulation, and further elevation of PGI\(_2\) levels upon oxygen restoration to tissue might have some protective value. Our results show such a ratio of cyclooxygenase products occurs in the isolated cerebral microvasculature in response to the "post-ischemic" incubation. Prolonged incubation in "N\(_2\)-CO\(_2\)" suggested that some imbalance in PG synthesis, i.e., a reduced ratio of PGI\(_2\) to PGE\(_2\) production, may occur. If such an imbalance does occur in vivo, it is likely that vasoconstrictor prostaglandin synthesis by other cell types (e.g., platelets, neural or glial elements) could play important roles. Various lines of reasoning may be advanced to account for the differential effects of the atmospheric manipulations of 6-keto PGF\(_{1\alpha}\) and PGE\(_2\) release. If there were more than one mechanism of arachidonic acid release perhaps regulated by different phospholipases, different patterns of PG production might occur. A recent report by Lysz and Needleman indicated two distinct forms of cyclooxygenase in brain tissue.\(^2\)\(^9\) These authors observed differential rate of and termination of PGE\(_2\) production in rabbit medulla; microsomes incubated with \([^{3}H]\) arachidonic acid. PGE\(_2\) levels rose rapidly and abruptly plateaued while PGF\(_{2\alpha}\) levels continued to rise for longer periods. The elimination of several possibilities to explain the differential rate (i.e., deficiency of substrate or cofactor etc.) supported the existence of two distinct cyclooxygenase types (e.g. platelets, neural or glial elements) which could play important roles. Various lines of reasoning may be advanced to account for the differential effects of the atmospheric manipulations of 6-keto PGF\(_{1\alpha}\) and PGE\(_2\) release. If there were more than one mechanism of arachidonic acid release, perhaps regulated by different phospholipases, different patterns of PG production might occur. A recent report by Lysz and Needleman indicated two distinct forms of cyclooxygenase in brain tissue.\(^2\)\(^9\) These authors observed differential rate of and termination of PGE\(_2\) production in rabbit medulla; microsomes incubated with \([^{3}H]\) arachidonic acid. PGE\(_2\) levels rose rapidly and abruptly plateaued while PGF\(_{2\alpha}\) levels continued to rise for longer periods. The elimination of several possibilities to explain the differential rate (i.e., deficiency of substrate or cofactor etc.) supported the existence of two distinct cyclooxygenases. The data from this series of experiments indicates that oxygen tension may differentially affect prostaglandin synthesizing activities leading to PGI\(_2\) versus PGE\(_2\) production. A possible means whereby such regulation could occur might be selective activation of a phospholipase.

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