Prostaglandin Synthesis and Oedema Formation During Reperfusion Following Experimental Brain Ischaemia In the Gerbil

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SUMMARY Regional brain tissue prostaglandin (PG) levels have been measured during ischaemia produced by bilateral carotid occlusion for 1 hour and following restoration of flow. In the normal gerbil, the frontal cortical levels of PGF₂α were: 6.7 ± 1.3 pg/mg and for PGE₂: 6.4 ± 1.1 pg/mg of brain tissue protein. Following 1 hour of ischaemia PGF₂α rose to 50.4 ± 8.3 pg/mg whilst there was only a slight rise in PGE₂ (10.7 ± 1.6 pg/mg). Post ischaemic values for parietal and occipital areas were somewhat higher, but showed the same trend. Within 15 minutes of the restoration of flow there was a massive increase in PGF₂α levels which reached a peak at 2 hours (300 pg/mg) and then subsided to control values. PGE₂ levels did not change for the first 30 minutes of recirculation, but then rose for the rest of the period of observation. The pattern of cytotoxic oedema resembled PGF₂α closely while the Evans blue staining (vasogenic oedema) was similar in time to the PGE₂ pattern.

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THE DISRUPTION OF CELLS due to trauma or ischaemia may release a wide variety of compounds causing profound vascular effects. The breakdown of the membrane also produces vasoactive compounds and it has been suggested that the release of polyunsaturated fatty acids (PUFAs) or their metabolites may be involved in the development of oedema.1 In previous work we have shown that pretreatment of our gerbil model with a cyclooxygenase inhibitor, indomethacin, which inhibits arachidonic acid metabolism, significantly reduced the amount of oedema accumulating in severely ischaemic brain tissue.2 This only occurred at cerebral blood flows below which a massive efflux of potassium has been demonstrated and has been taken as evidence of cell membrane disruption.3

The effect of restoration of flow has also been studied,4 and we have shown that there is an acceleration of water accumulation in severely ischaemic brain following recirculation. This precedes the leakage of protein linked markers such as Evans blue. The source of the water is the vascular system but the initiating mechanisms for the water accumulation or leakage of Evans blue are far from clear. If the products of cell disruption are implicated in ischaemic oedema, may they also be involved during reperfusion? Might these compounds produced by ischaemia interact with the recirculating blood and further aggravate the situation?

The purpose of this study has been to measure the production of prostaglandins (PG) during ischaemia and recirculation to determine if there is a time relationship with oedema formation during reperfusion.

Methods and Materials

A) Surgical Preparation

Adult male gerbils (Meriones unguiculatus, 50–60g) were used in all experiments. Surgical preparations, carotid ligation, thoracotomy, “kill” perfusion and dissection of brain were performed in an identical fashion in all animals, as previously described.5 Animals were anaesthetised with pentobarbital (60 mg/kg) intraperitoneally and a microscopic dissection of the neck performed to allow simultaneous occlusion of both carotid arteries with Scoville aneurysm clips. Exactly 1 hour after bilateral carotid occlusion, aneurysm clips were removed and the cerebral circulation restored for varying periods of time for up to 3 hours. A thoracotomy involving removal of the sternum allowed access to the heart. This enabled the “kill” perfused technique to be effected; the right atrium was opened, the descending aorta occluded and a cannula inserted into the left ventricle. A combination of heart beat and hydrostatic pressure allowed perfusion of the upper half of the gerbil body using a cold solution of indomethacin/saline (10 μg/ml) for a period of 2 minutes. The brain was then rapidly removed and dissected in a Petri dish containing the same solution, the whole procedure taking less than 1 minute. The frontal lobes were dissected anterior to the optic chiasm with removal of the olfactory bulbs. Parietal cortex was dissected external to the lateral ventricles in order to exclude any deep grey matter. The portion of the occipital lobe chosen was the posterior-inferior part of the cerebral hemisphere. Samples from all areas were cut cleanly with a sharp scalpel and then rapidly frozen in liquid nitrogen. Similar sample sizes were obtained from each area, approximately 70 mg in weight, variation being avoided as far as possible in order to allow similar dilutions for radioimmunoassay and protein estimations.

The technique of “kill” perfusion was considered to be important in order to biochemically “freeze” (vida infra) the synthesis of PGs by the tissue during sampling. For the ischaemia model, the clips were not removed from the carotid arteries, unlike the reperfusion model.
B) Prostaglandin Extraction and Assay

Samples were sonicated using an M.S.E. ultrasonicicator at maximum amplitude for 10 seconds in an ice-cold solution of citric acid (pH 3.5), and indomethacin (10 µg/ml). Addition of 1000 dpm of (H) PGF$_{2\alpha}$ (180Ci/mmol) and (H) PGE$_2$ (120Ci/mmol) (Amersham International U.K.) were employed as internal standards. The samples were extracted twice in 8 volumes of cyclohexane:ethylacetate (1:1 v/v) and after centrifugation at 2000 g, the solvent layers were pooled and stored at $-20^\circ$C under nitrogen for up to 24 hours. The solvents were evaporated to dryness under reduced pressure and the residue taken up in 1 ml cyclohexane:ethylacetate:methanol (60:40:0.2).

Due to the known interference of nonesterified fatty acids in radioimmunoassays, it was necessary to separate the PGs by column chromatography on activated silicic acid (0.5g, 100 mesh Mallinkrodt) using the following solvents: —

1) 5 ml cyclohexane:ethylacetate (60:40), to remove nonpolar lipids; (2) 10 ml cyclohexane:ethylacetate:methanol(60:40:2), to elute PGE$_2$; (3) 5 ml (60:40:20), to elute PGF$_{2\alpha}$.

The various fractions were again evaporated under vacuum, taken up in 1 ml 0.1M phosphate buffer at pH 7.4 (0.9% NaCl), 1% gelatin and 1% Sodium azide w/ v and kept at 4°C for 2 hours. Recovery estimated by internal standards was 63 $\pm$ 10 (mean $\pm$ S.D.) for both PGF$_{2\alpha}$ and PGE$_2$, through the whole extraction and separation procedure. Both were measured by radioimmunoassay at three different dilutions (1:10, 1:150, 1:100) in order to utilise the linear portion of the standard calibration curves. Anti PGE$_2$ and PGF$_{2\alpha}$ antisera were purchased from Sigma (London) and found to be of high specificity and low cross-reactivity, which was further enhanced by the separation procedure. The specificity of the determination with respect to cross-reactivity between the PGF$_{2\alpha}$ and PGE$_2$ was as follows. Recovery for PGE$_2$ in the 60:40:2 fraction by the radioimmunoassay was 95%, and 2% for PGF$_{2\alpha}$, whereas 90% recovery was obtained for PGF$_{2\alpha}$ with the 60:40:20 fraction, with no binding for PGE$_2$. After incubation and charcoal separation, the unbound radioactivity ligands were counted by liquid scintillation on a Beckman LT 3150, with a counting efficiency of 56% for tritium. Protein estimations were carried out by the method of Lowry et al., with bovine serum albumin as standard. Results are expressed in pg/mg protein, rather than pg/g wet weight to exclude errors caused by alterations of brain water content following ischaemia.

Validation of “Biochemical Freezing” Technique

The validation was carried out on 3 groups of control (unligated) animals. All animals were perfused with sterile solutions (ice-cold) to avoid pyrogen stimulated PG synthesis. The first group of animals were not perfused, and the brain sampled with blood present within the vasculature. The second group of animals were perfused as outlined above with saline alone, and the final group perfused with indomethacin/saline. The PGs were extracted and assayed as stated above.

Regional Cerebral Blood Flow (rCBF)

rCBF was evaluated by the hydrogen clearance technique which has been described in detail elsewhere.

Measurement of Water Content and Vasogenic Oedema

Brain water was estimated in other series of similarly prepared gerbils sacrificed at identical points during reperfusion. The specific gravity technique was employed, with the modification of Marmarou et al. This method estimates changes in total brain water, and assumes tissue solids are constant.

The escape of Evans blue from the vascular compartment was taken as evidence of protein leakage into the tissues and used to indicate “vasogenic oedema”. In another group of animals, 2% Evans blue (EB) in buffered saline (0.05 ml) was injected intraperitoneally 1 hour prior to carotid occlusion and followed by sacrifice at the same time points of reperfusion as for PG content measurements. Evans blue extravasation was estimated visually by sacrificing the animals following various periods of reperfusion and after coronal sectioning of their brain.

Results

Validation of “Biochemical Freezing” Technique

Table 1 demonstrates the effect of kill perfusion with either saline or indomethacin/saline on PG levels as compared to control animals. Saline perfusion alone reduces the PG levels in the gerbil brain but not to the same extent as that seen with indomethacin/saline perfusion. The control results were lower than those reported by others and indicates the importance of the vascular compartment as a source of PGs.

Brain PG Content and rCBF Following Bilateral Carotid Ligation

Table 2 summarizes the effect of bilateral carotid ligation for 1 hour on prostaglandin content and rCBF in various regions of brain. PGF$_{2\alpha}$ values increased by up to sixfold in the occipital regions with smaller increases in the frontal and parietal regions. While there was some increase in PGE$_2$ levels during ischaemia, it was not of the same order as that found with PGF$_{2\alpha}$.

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Table 1 Biochemical Freezing of Prostaglandin Synthesis

<table>
<thead>
<tr>
<th></th>
<th>PGF$_{2\alpha}$</th>
<th>PGE$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pg/mg) (%)</td>
<td>(pg/mg) (%)</td>
</tr>
<tr>
<td>Control</td>
<td>16.3 100</td>
<td>17.1 100</td>
</tr>
<tr>
<td>Saline</td>
<td>10.0 61</td>
<td>16.9 98</td>
</tr>
<tr>
<td>Indomethacin and saline</td>
<td>4.8 26</td>
<td>8.0 47</td>
</tr>
</tbody>
</table>

Brain cortical PG levels in control animals and those perfused with either saline alone or indomethacin/saline (10 µg/ml). The perfusion solution was maintained at 4°C. There was a significant decrease in both classes of PGs following perfusion especially with indomethacin/saline.

Absolute results expressed in pg/mg tissue protein.

Relative results expressed as percentage with control assumed at 100%.

n = 3 for each group.
rCBF was estimated by the hydrogen clearance technique, showed a marked reduction following bilateral carotid occlusion for 1 hour. There was a gradation in ischaemic flow from frontal to the occipital regions.

**Time Course of PG Synthesis During Reperfusion**

The effect of bilateral carotid occlusion in the gerbil, for 1 hour following by reperfusion for periods of up to 3 hours on PGF2α and PGE2 content is shown in figure 1.

There was no significant change in the levels of PGF2α during the initial 15 minutes of reperfusion, but then they became elevated for 2 hours and returned back to "control" values in the third hour. PGE2 values did not change significantly for 30 minutes, after which they became elevated and continued to rise during the 3 hours of reperfusion.

**Changes in Specific Gravity Measurements During Reperfusion**

Figure 2 depicts the changes in specific gravity (SG) measurements during reperfusion following 1 hour of ischaemia represented by the parietal region. The profiles were similar in the other areas; frontal and occipital regions.

During 1 hour of ischaemia, all areas showed a significant fall in SG (increase in oedema). There was, further, a more rapid accumulation of oedema within 5 minutes of clip removal. The fall in SG was most marked by 60 minutes. All areas showed a return towards normal SG in the third hour of reperfusion.

**Evans Blue (EB) Staining During Reperfusion**

The development of vasogenic oedema as marked by EB staining during reperfusion is summarised in table 3; number of animals with positive EB extravasation as a ratio of total within each group. The earliest EB staining was seen at 60 minutes of reperfusion. 50% of the brains were stained with EB by 120 minutes, increasing in degree at 180 minutes. The dye was most frequently bilateral and symmetrical, staining the deep grey structures.

**Discussion**

During the process of phospholipid deacylation in ischaemia, the release of arachidonic acid AA may be monitored by measuring the metabolites, namely the PGs. Hence changes in PG levels are possible indications of membrane degradation and therefore reflect changes in permeability and transport capabilities. This study concentrates only on the cyclooxygenase products of AA metabolism and it does not rule out the possibility that other AA metabolites may also be involved, such as those formed via the lipoxygenase pathways. It has been demonstrated that AA products such as leukotrienes and, to a lesser extent, PGE2 do affect vascular permeability.

Measurement of tissue PG levels may be complicated by the rapid synthesis of PGs during the process of sampling, and special precautions are required to prevent this artefact. In this study we describe a method of "biochemically freezing" the PG levels in situ during sampling, and the "kill" perfusion technique which we have also described has provided reliable and reproducible results showing significant differences in relatively small changes in PG levels with minimal error arising from tissue handling during the sampling procedure (see table 1). The technique "washes out" the vascular compartment and we consider that most of the PGs measured are derived from brain tissue during the ischaemic insult.

During periods of ischaemia the increased synthesis, as evidenced by high tissue levels of PGs, probably indicates an increased availability of substrate, (AA), derived from the breakdown of membranes. Metabolism of AA to PGs via cyclic endoperoxides is dependent upon a number of factors. These include oxygen availability and hence blood flow. The differential and much smaller rise in PGE2, as compared to the six-fold increase in PGF2α may itself be due to
several factors, one of which may be differences in the normal Michaelis-Menten kinetics of the PG isomerasases involved (vida infra). Another possibility may be the distribution of these enzymes within the brain tissues. Wolfe\textsuperscript{13} reported a similar differential synthesis of PGE\textsubscript{2} and PGF\textsubscript{2\alpha} in brain homogenates and tissue slices. PGE\textsubscript{2} synthesis reached completion within 15 minutes of incubation whereas PGF\textsubscript{2\alpha} synthesis continued for up to 1 hour. Our data during ischaemia are similar but the results following reperfusion could not be explained in this fashion. With the restoration of cerebral flow there is a 40-fold increase in PGF\textsubscript{2\alpha} which reaches peak values within 1 hour, thereafter declining to levels found with ischaemia alone (Figure 1). With reperfusion PGE\textsubscript{2} levels do rise by tenfold but only after a delay of 2 hours. It might be postulated that the relatively larger response of PGF\textsubscript{2\alpha} is due to the sensitivity of PGE-isomerase to high substrate concentrations,\textsuperscript{14} and it might also be argued that if our observations during ischaemia alone had extended over the same time as the recirculation experiments then a similar pattern would have emerged. As the amount of AA was depleted, so the inhibition of the enzyme would have been reduced, thus allowing greater production of PGE\textsubscript{2}. At the same time PGF\textsubscript{2\alpha} synthesis would continue to fall due to a decrease in substrate concentration, following a normal Michaelis-Menten pattern of enzyme kinetics. An argument against this explanation is found in Gaudet and Levine's\textsuperscript{15} work with 1 to 30 minutes occlusion in gerbils, in which they found only a transient increase in PGE\textsubscript{2} and no progressive trend as we have shown. An alternative explanation might be the selective catabolism of PGE\textsubscript{2}, but this, too, is unlikely due to the low PG-dehydrogenase activity in brain compared with other tissues.\textsuperscript{16} A third explanation could be that of the "washout" of PGs into the vascular compartment either by simple diffusion or by a saturable facilitated transport mechanism.\textsuperscript{17} This, however, could not account for the results obtained during ischaemia alone, due to reduced blood flows. What is increasingly emphasised in ischaemic damage to the nervous system is that the depth of ischaemia and the duration of the insult are important.\textsuperscript{18} It appears that a 5 or 15 minute interruption of carotid flow can be well tolerated with practically no mortality or morbidity. A 30 minute occlusion followed by reperfusion has a mortality of 40\% at 24 hours,\textsuperscript{18} whereas occlusion for

\begin{table}[h]
\centering
\caption{Evans Blue Extravasation during Reperfusion}
\begin{tabular}{lcc}
\hline
 & 1 hr & 2 hrs & 3 hrs \\
\hline
Evans blue & 1/9 & 5/10 & 4/8 \\
\hline
\end{tabular}
\end{table}

Evolution of EB leakage following 60 minutes of bilateral cerebral ischaemia and reperfusion. Results expressed as ratio of animals with EB extravasation over the total gerbils per group. There is no sign of EB until the first hour but increases rapidly to 50\% by the second hour. The EB seen in animals at the third hour is greater in degree as compared to those at two hours of reperfusion.
1 hour produces a 93% mortality over the same period. In the model we have chosen, there is extensive infarction in the few survivors while in the 1 to 30 minute model used by Gaudet and Levine there may be minimal disruption of cerebral architecture. Our results therefore are not at variance with theirs but represent the other end of an ischaemic spectrum. Nevertheless, there are significant differences in the results of our two groups, and other explanations need to be sought.

With respect to Evans blue studies, as shown by others and ourselves, there is no leakage of dye from the vascular compartment following a 5 minute occlusion and only an occasional example with 15 minute carotid interruption at 24 hours of reperfusion; but following 60 minutes of ischaemia 50% show EB staining of tissues within 120 minutes of reperfusion (see table 3). This would suggest a profound alteration in the blood brain barrier (BBB) following ischaemia of this length and depth during which intravascular substances will leak out into the damaged tissue, leading to situations different from those obtained in the shorter occlusion times. It must be borne in mind that EB staining provides a gross marker rather than a sensitive one, therefore, its incidence would only be expected in extensive BBB damage, although disruption to the vasculature has been shown to occur to a lesser degree much earlier over a larger area using a sensitive marker such as sucrose extravasation. We were impressed by the similar time scales for the elevation of PGE$_2$ and the appearance of EB staining, and we postulate that the phenomena may be causally related. PGE$_2$ is a major product of the microcirculation in bovine brain, and Pickard has shown that PGE$_2$ represents up to 50% of AA metabolism in canine cerebral arteries. It may be that in our model of prolonged and profound ischaemia there is vascular endothelial damage which gives rise to PGs when reperfusion occurs. The vascular damage will then result in breakdown of the BBB and intravascularly generated PGs will "leak" into brain tissue and be measured by our technique. The question then arises as to whether the breakdown of the BBB and endothelial PG generation are two separate effects of the ischaemic insult, or whether they are related. Clearly, more work will be required to substantiate this relationship.

Our main conclusion so far, is that following the restoration of flow in severely ischaemic brain there is a rapid production of PGE$_2$, the profile of which resembles that of brain water content over the same period in the same model. By contrast, the increase in PGE$_2$ is delayed but progressive, and is not found in shorter periods of ischaemia which in turn are not mortal in the gerbil and not associated with Evans blue extravasation.

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### References

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