EFFECT OF INTRAVENOUS EICOSAPENTAENOIC ACID ON CEREBRAL BLOOD FLOW, EDEMA AND BRAIN PROSTAGLANDINS IN ISCHEMIC GERBILS

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Abstract

Eicosapentaenoic acid is converted by cyclo-oxygenase to the prostacyclin, PGI_3. Consequently eicosapentaenoic acid might protect the brain from the impairment in cerebral blood flow that follows temporary cerebral arterial occlusion. We studied the effect of 90% pure eicosapentaenoic acid, given intravenously, on cerebral blood flow, brain water and prostaglandins after ischemia in gerbils. Ischemia was produced by bilateral carotid occlusion for 15 min followed by reperfusion for 2 h. In experimental gerbils, 0.833 mg or 0.167 mg of eicosapentaenoic acid (Na salt) was given intravenously followed by a continuous infusion of 1 mg h^{-1}. Control gerbils were given 0.167 mg of linoleic acid (Na salt) intravenously followed by a continuous infusion of 1 mg h^{-1} or a saline infusion. Regional cerebral blood flow was measured by the hydrogen clearance method and brain water by the specific gravity technique. Brain diene prostaglandins were measured by radioimmunoassay. In control gerbils cerebral blood flow decreased significantly during reperfusion and remained depressed after 2 h of reperfusion. In eicosapentaenoic acid treated gerbils blood flow decreased initially but after 2 h of reperfusion blood flow was significantly higher than in control gerbils. Brain edema and brain diene prostaglandins were not significantly different between control and experimental groups.

Our study indicates that eicosapentaenoic acid, given intravenously, improves cerebral blood flow after ischemia and reperfusion. We speculate that this effect may be due to the formation of the prostacyclin, PGI_3.

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Introduction

A therapeutic potential for eicosapentaenoic acid (EPA) was suggested by the findings of Dyerberg (1) who noted the high content of EPA in the maritime diet of Greenland Eskimos. EPA was suggested as an important factor in the low incidence of myocardial infarction in Eskimos (1-3). Dyerberg indicated that EPA could have an antiaggregatory action on blood platelets due to competitive inhibition of TXA_2 synthesis. This is in accord with the observation that EPA is bound to the enzyme cyclo-oxygenase from sheep vesicular gland with affinities (1.7 to 15 μM) equal to or greater than the Km value for arachidonate. EPA was thus suggested to be an effective competitive inhibitor (4). Moncada and Vane (5), however, regarded the protective effect as being due to the ability of the vessel wall to utilize EPA to make a Δ^{17} prostacyclin (PGI_3), a vasodilator and inhibitor of platelet aggregation, and an accompanying thromboxane A_3, which is not active. In support of this concept, Dyerberg (6) recently reported that human umbilical blood vessel walls transform EPA to PGI_3.

We previously reported that dietary supplements of menhaden oil, which is 17% EPA by weight, prevented impaired cerebral blood flow (CBF) and edema in ischemic gerbils (7). In this study the effect of 90% pure EPA, given intravenously, on CBF, edema and brain diene prostaglandins after ischemia and reperfusion is reported.

Materials and Methods

1) Surgical Procedure

Male mongolian gerbils (Meriones unguiculatus) weighing 50 to 77 g were anesthetized with ketamine (75 mg kg^{-1}) and xylazine (20 mg kg^{-1}) injected intraperitoneally. Anesthesia was maintained with ketamine 37.5 mg kg^{-1} and xylazine 10 mg kg^{-1} (i.p.) every 20 to 40 min as required for the duration of the experiment. A midline neck incision was made and the trachea was cannulated with a PE-90 polyethylene catheter. PE-10 polyethylene catheters were inserted into the left femoral artery and vein. The arterial catheter was connected to a blood pressure transducer and systemic arterial blood pressure was continuously monitored. The venous catheter was connected to a 1 cc glass syringe attached to a constant infusion pump. Arterial blood samples were obtained for blood gas analysis immediately after the last CBF measurement. Blood samples were not obtained prior to the last CBF to avoid the effects of phlebotomy on CBF and edema in ischemic gerbils (8). The right and left common carotid arteries were exposed in the neck with the aid of magnification. Both carotid

arteries were occluded with Mayfield clips for 15 min. The clips were then removed to restore cerebral circulation. In sham operated animals the carotid arteries were exposed but not occluded. In animals that had CBF measurements burr holes were made bilaterally over the frontal and parietal cortex. Four Teflon coated platinum electrodes (125 µm diameter) were inserted stereotactically 0.5 mm into the cerebral cortex. Exposed cortex around the electrodes was covered with moist Gelfoam and the electrodes were fixed in place with acrylic cement. A silver/silver chloride reference electrode was placed subcutaneously in the right leg.

2) Measurements

Regional CBF was measured by the hydrogen clearance technique (9). Brain specific gravity measurements were made using a bromobenzene-kerosene density gradient column as previously described (7).

In gerbils that were used for prostaglandin determinations the brain was removed in less than 1 min and the left cerebral hemisphere was placed in pentane, cooled in dry ice. The frozen brains were weighed and placed in a 30 ml tissue grinding tube kept on ice. Aspirin-formic acid solution (1 ml) was added to the frozen tissue. Aspirin-formic acid solution was prepared by dissolving 0.207 g acetylsalicylic acid in 100 ml of 0.75% saline. Aliquots (4.5 ml) were frozen in plastic vials and stored. Prior to use, 0.5 ml of 88% formic acid was added to a vial containing the frozen aspirin solution and the contents were allowed to thaw slowly on ice. The tissue was slowly homogenized using a serrated-tip Teflon pestle. The homogenized tissue was poured into a test tube. The glass grinding tube was rinsed with 4 ml of ethyl acetate which was then added to the homogenized brain tissue in the test tube. The homogenized tissue and ethyl acetate were mixed for 30 sec with a vortexer and centrifuged for 5 min at 2500 rpm. The top layer was removed and dried under an airstream. Three milliliters of 0.1 M phostate buffered saline was added to the sample followed by 3 ml of petroleum ether (30-60°C). The sample was mixed for 60 sec and centrifuged as before. The sample was then stored at -20°C until prostaglandin assays were performed.

PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 were determined by radioimmunoassay using ^{3}H-labelled compounds from New England Nuclear Corp. (10-12). The antibodies were from Upjohn except for 6-keto-$PGF_{1\alpha}$, which was determined with a kit (New England Nuclear). Dextran-coated charcoal was used to separate bound from free ligand. The limits of sensitivity were 2 pg/tube (PGE_2), 1.3 pg/tube ($PGF_{2\alpha}$), 4.5 pg/tube (6-keto- $PGF_{1\alpha}$), and 0.7

pg/tube (TXB_2). The cross-reactivity of the antibodies to the triene prostaglandins TXB_3 and Δ^{17} 6-keto $PGF_{1\alpha}$, has not been determined. Cross-reactivity of the antibody for PGE_2 to PGE_3 was 7.8%. The cross-reactivity of the antibodies to other related prostaglandins were low. The percent of extraction recovery of prostanoids were 98.4% for PGE_2, 99.1% for $PGF_{2\alpha}$, 96.8% for TXB_2 and 96.7% for 6-keto $PGF_{1\alpha}$. The background values for the mean extracted H_2O blank and PBS buffer respecitvely were 12 and 36 pg/ml for PGE_2, 16 and 16 pg/ml for $PGF_{2\alpha}$ and 8 and 7 pg/ml for TXB_2. Background values were 0 for 6-keto $PGF_{1\alpha}$. The inter and intra-assay percent coefficient of variation, respectively, were 10.5 and 5.8 for PGE_2, 8.7 and 2.0 for $PGF_{2\alpha}$, 4.9 an 4.3 for 6-keto $PGF_{1\alpha}$ and 12.9 and 3.8 for TXB_2.

3) Preparation of Fatty Acids for Injection

The EPA (all cis-5,8,11,14,17-eicosapentaenoic acid, 90% pure) was obtained as the ethyl ester from Nippon Kagakushiryo Co, Hokkaido, Japan. The contaminants inluded: 1.1% 18:4 (n-3), 1.2% 20:4 (n-6), 0.7% 20:4 (n-3) and 1.9% 22:6 (n-3)

The ester was saponified under nitrogen, using the valve of Raymond Firestone (Aldrich Chemical Co.) to remove air. About 250 mg were refluxed for 1 h in a 1-piece condenser flask with 7 ml of 0.3 M NaOH in methanol-water (13). The reaction mixture was extracted with 5 ml of hexane, which removed about 2 mg of oil, then acidified with HCl and extracted with three 5-ml portions of hexane. The pooled extracts were washed with water, dried with sodium sulfate, and evaporated to dryness with a stream of nitrogen in a Thunberg tube. To the acid was added enough 0.3 M NaOH to bring the pH to about 8.3 and enough isotonic saline to bring the concentration of the acid to 10 mg ml^{-1}. The air in the tube was removed with the Firestone valve and the mixture was warmed and sonicated to produce a cloudy suspension.

Portions of the suspension (0.4 ml) were dispensed into test tubes made from 0.6 mm O.D. Pyrex tubing. These were frozen in dry ice, evacuated with a high-vacuum pump, and sealed. The tubes were stored at -20°C until use, when they were opened and diluted with an equal volume of alkaline saline (10 ml saline + 0.2 ml of 0.3 M NaOH) to yield a clear solution, pH about 8.5.

For comparison, similar suspensions were prepared from linoleic acid (cis,cis-9,12-octadecadienoic acid from Applied Science), with omisson of the saponification step. The sealed tubes were color coded (two colors for each acid) for blind testing.

4) Experimental Protocol

In all ischemic animals both carotid arteries were occluded for 15 min. In 30 gerbils that had CBF and brain water determinations, after 15 min of occlusion the clips were removed and cerebral circulation restored for 2 h. CBF measurements were taken prior to occlusion, during occlusion and 5 min, 30 min, 60 min and 120 min after reperfusion. The brains were then immediately removed for specific gravity determinations.

In 20 gerbils, 0.0334 ml min^{-1} of 0.9% saline or EPA (0.835 mg) in saline was infused starting 5 min prior to carotid occlusion. The infusion was continued during occlusion and reperfusion at 0.2 ml h^{-1} (1.0 mg h^{-1}) for 135 min. In a second experiment 0.167 mg of linoleic acid (n=5) or 0.167 mg of EPA (n=5) was infused over 1 min starting immediately after carotid occlusion and was continued at a rate of 1 mg h^{-1} for 135 min.

Brain prostaglandins were determined in 40 gerbils. In sham operated EPA gerbils 0.167 mg min^{-1} of EPA (n=10) was infused for 5 min followed by a constant infusion of 1 mg h^{-1}. After 65 min of infusion the brains were removed and assayed for prostaglandins. In ischemic EPA gerbils 0.167 mg min^{-1} of EPA (n=10) was infused for 5 min followed by an infusion of 1 mg h^{-1} for 30 min. After 30 min of infusion both carotids were occluded for 15 min followed by 15 min of reperfusion. The infusion of EPA (1 mg h^{-1}) continued during occlusion and reperfusion. Control gerbils (n=20) were given no infusions. In control ischemic gerbils ischemia was produced for 15 min followed by 15 min of reperfusion. After 15 min of reperfusion in both control and EPA gerbils the brains were removed for prostaglandin analysis.

5) Statistical Analysis

An analysis of variance was performed between groups to determine significant differences between CBF, edema and prostaglandin levels.

Results

Prior to carotid occlusion, in saline control gerbils, regional CBF was 27.4 ml 100 g^{-1} min^{-1}. During bilateral carotid occlusion, CBF was less than 5 ml 100 g^{-1} min^{-1}. There was a short period of hyperemia 5 min after reperfusion.

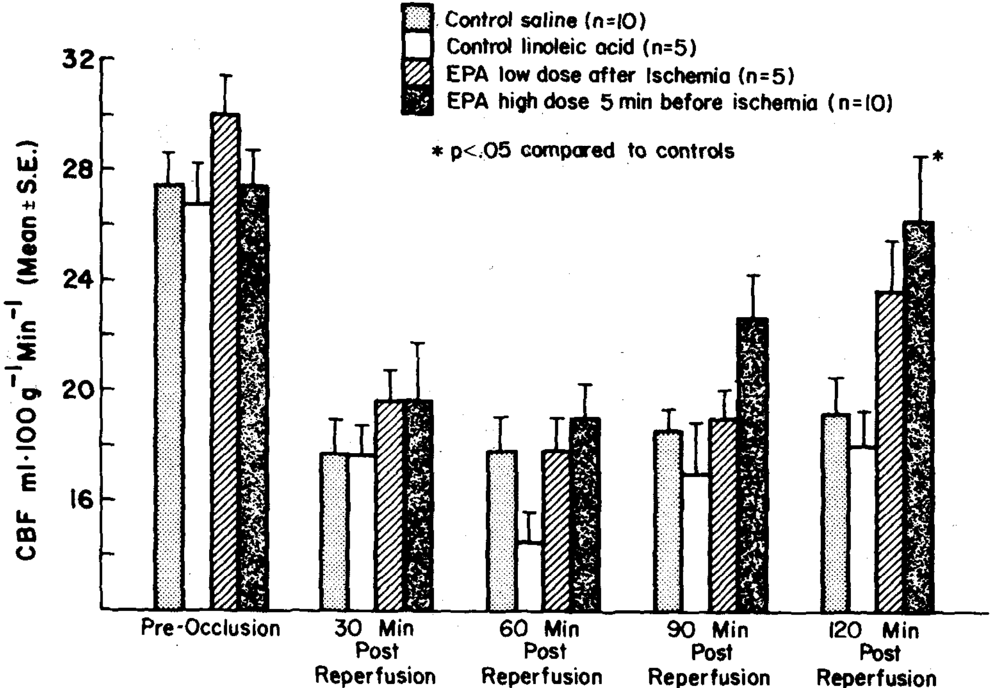

Figure 1. CBF prior to carotid occlusion and during 2 h of reperfusion after ischemia. Low dose EPA animals received immediately after carotid occlusion 0.167 mg of EPA over 1 min followed by an infusion of 1 mg h^{-1}. In high dose EPA animals, 0.835 mg of EPA was infused over 5 min prior to occlusion. The infusion was continued during occlusion and reperfusion at 1 mg h^{-1}.

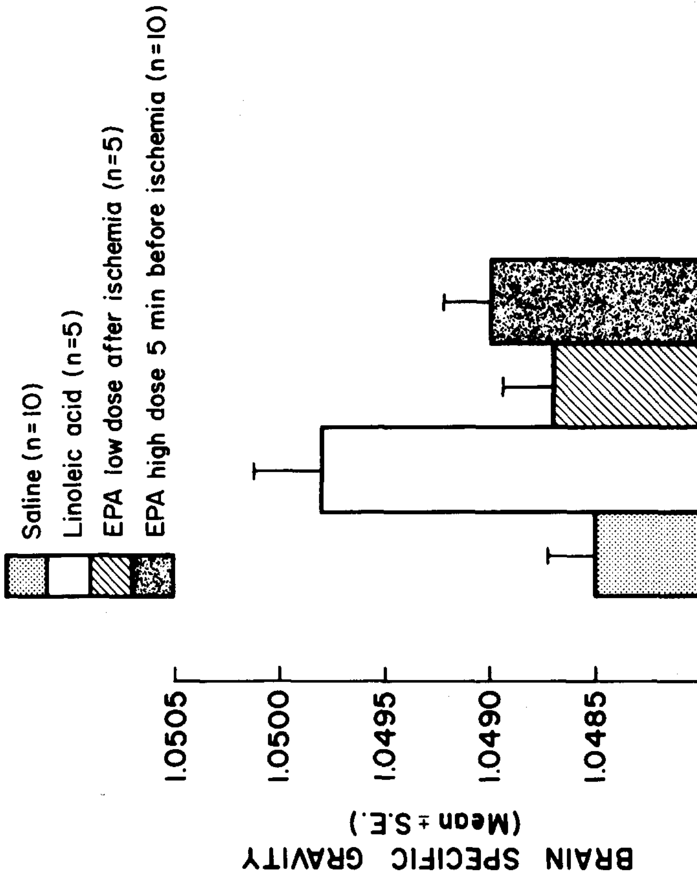

Figure 2. Brain specific gravity after 15 min of carotid occlusion and 2 h of reperfusion.

TABLE I

BRAIN PROSTAGLANDINS (PG/MG BRAIN) IN CONTROL SHAM AND ISCHEMIC GERBILS AND IN EPA INFUSED SHAM AND ISCHEMIC GERBILS. MEAN ± S.D.

	6-Keto $PGF_{1\alpha}$		TxB_2	
	Sham (n=10)	Ischemic (n=10)	Sham (n=10)	Ischemic (n=10)
Control	1.93 ± 1.44	6.49 ± 2.35	1.24 ± .85	2.94 ± .83
EPA	4.32 ± 1.99	8.03 ± 2.57	2.06 ± .79	3.08 ± 5.7

	PGE_2		$PGF_{2\alpha}$	
	Sham (n=10)	Ischemic (n=10)	Sham (n=10)	Ischemic (n=10)
Control	16.87 ± 9.66	32.96 ± 18.10	7.18 ± 5.45	40.27 ± 11.66
EPA	40.84 ± 14.36	46.39 ± 11.3	14.38 ± 8.43	44.71 ± 9.99

After 30 min of reperfusion CBF fell to 17.7 ml 100 $g^{-1}min^{-1}$ and remained depressed at 19.2 ml 100 g^{-1} min^{-1} after 2 h of reperfusion. In gerbils infused with 0.835 mg of EPA 5 min prior to ischemia followed by constant infusion during ischemia, CBF decreased from 27.4 ml 100 g^{-1} min^{-1} to 19.6 ml 100 g^{-1} min^{-1} initially but after 2 h of reperfusion CBF was improved to 26.2 ml 100 g^{-1} min^{-1}, significantly higher than control saline gerbils at $p < 0.05$ (Fig. 1). In gerbils infused with 0.167 mg of EPA after ischemia, followed by constant infusion, CBF decreased from 30.0 to 17.8 but improved after 2 h of reperfusion to 23.7 ml 100 g^{-1} min^{-1}. Animals infused with linoleic acid had blood flows similar to saline infused gerbils (Fig. 1).

Brain edema was less in gerbils infused with EPA or linoleic acid compared with gerbils infused with saline (Fig. 2). The differences between the 4 groups were not significant at the 0.05 level.

Brain levels of PGE_2, $PGF_{2\alpha}$, TXB_2 and 6-keto $PGF_{1\alpha}$ were higher after ischemia in gerbils infused with EPA. The differences were not significant, however (Table 1).

Blood pressure and arterial blood gases remained within physiologic limits and were similar in all groups. There was no decrease in systemic blood pressure after EPA infusion .

Discussion

There is growing evidence that a variety of oxidized products of arachidonate (thromboxane A_2, prostacyclin I_2, leukotrienes) are important in ameliorating or potentiating ischemic injury. During severe ischemia there is a 20 to 40 fold increase in the tissue content of free arachidonate (14,15). When blood flow is re-established, restoring tissue oxygen, arachidonic acid is converted to prostaglandins, prostacyclin and thromboxane (16,17). EPA is structurally similar to arachidonate and it may prevent platelet TXA_2 production by competitive inhibition of arachidonate oxidation by cyclo-oxygenase (18). EPA is itself oxidized by cyclo-oxygenase to a Δ^{17} prostacyclin, PGI_3 (5,6), which is an inhibitor of platelet aggregation, and to TXA_3, which itself is inactive. TXA_3 may have a beneficial effect, however, by competing with TXA_2 for receptor sites.

Intravenous infusion of EPA significantly improved post-ischemic CBF after 90 min of infusion. Although we did not measure Δ^{17} 6-keto $PGF_{1\alpha}$, we speculate that improved post-ischemic CBF may be due to the formation of PGI_3. Further, intravenous infusion of EPA may, theoretically, result

in a selective increase in PGI_3 in ischemic tissue. Culp (19) reported that EPA is a poor substrate for cyclo-oxygenase at peroxide levels which occur in non-ischemic tissue. Increasing peroxide tone in an incubate containing cyclo-oxygenase increased the conversion of EPA considerably. Accordingly, in areas of ischemia where peroxide levels are higher, the formation of PGI_3 may be increased. Moncada (5,20) has also emphasized the importance of interactions between platelets and the vessel wall. It was suggested that platelets adhering to the vessel wall could possibly feed the vessel enzyme, prostacyclin synthetase, with endoperoxides, thereby enabling the vessel to produce more prostacyclin. In the ischemic area, platelets adhering to cerebral vessels could convert the EPA to cyclic endoperoxides and transfer these endoperoxides to the vessel wall where PGI_3 is formed. In non-ischemic tissue, without platelet adhesion to the vessel wall and with lower peroxide levels, the formation of PGI_3 may be less. The absence of systemic hypotension after infusion of a large dose of EPA adds support to the suggestion that PGI_3 may be selectively formed in ischemic tissue.

Brain diene prostaglandin and thromboxane levels were higher, but not significantly, in animals infused with EPA. These findings are difficult to interpret for several reasons. First, EPA is converted by cyclo-oxygenase to triene prostaglandins, whereas arachidonate is the precursor of the diene prostaglandins. The cross-reactivity in our assay for PGE_2 with PGE_3 is relatively high, 7.8%. We have not determined the cross-reactivity for TXB_3 or Δ^{17} 6-keto $PGF_{1\alpha}$. Therefore, a large production of PGI_3 or TXA_3 may only be reflected as a slight increase in 6-keto $PGF_{1\alpha}$ or TXB_2 due to cross-reactivity in our assay. Conversely, inhibition of diene prostaglandin production by EPA may be masked by increased triene prostaglandins. Second, there is evidence (unpublished data in our laboratory) that the brain thromboxane measured by our assay does not entirely reflect the level of platelet thromboxane. There is recent evidence that the vessel wall itself is also a source of thromboxane (21). The formation of thromboxane from this source is relatively insensitive to aspirin (22) and, perhaps, to EPA. Third, our infused EPA contained a small amount of arachidonate which may have been converted into diene prostaglandins.

In a previous report (7) we noted that dietary supplements of menhaden oil, which is 17% EPA by weight, prevented brain water accumulation during the post-ischemic period. Intravenous infusion of EPA did not significantly reduce brain water accumulation. Prevention of brain edema by

EPA may require the incorporation of EPA into cell membranes, which can only be achieved by long term dietary supplements of EPA. The menhaden oil could also contain other polyunsaturated fatty acids which could have accounted for the reduced brain water accumulation. In this regard it is of interest that brain water accumulation was lowest, but not significantly, in gerbils infused with linoleic acid although CBF was not improved.

In conclusion, intravenous infusion of EPA improved post-ischemic CBF. We speculate that the increased CBF may be due in part to the production of PGI_3. Further investigation into the mechanism of this beneficial effect and its clinical applicability in ischemia is warranted.

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