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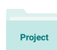

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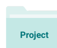

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RESEARCH NOTE

Dual inhibition of cyclooxygenase and lipoxygenase enzymes by human cerebrospinal fluid

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Abstract Thromboxane A_2 (TXA_2) formed in damaged brain tissue and after thromboembolism and subarachnoid haemorrhage is responsible for cerebral vasospasm. In the present study, we examined the effect of human cerebrospinal fluid (CSF) on the production of thromboxane-A_2 (TXA_2) and 12-hydroxy-eicosatetraenoic acid (12-HETE) by human blood platelets. CSF was drawn by lumbar puncture from normal healthy volunteers ($n = 17$) and samples judged to be normal after routine examination in the clinical laboratories and were used fresh. We found that CSF inhibited the production of TXA_2 and 12-HETE by blood platelets incubated with C^{14} labelled arachidonic acid (AA) in a concentration-related manner. Further biochemical analysis using proteolytic enzymes, gel filtration and membrane partition chromatography showed that the inhibitory activity was peptidic in nature and associated with a peptide of low molecular weight (1,400 Da). This study is the first to demonstrate that human CSF contains a dual inhibitor of cyclooxygenase (COX) and lipoxygenase enzymes in CSF.

Keywords Cerebrospinal fluid · Platelet · Arachidonic acid metabolism · Thromboxane-A_2 · 12-Hydroxy-eicosatetraenoic acid

Abbreviations

AA	Arachidonic acid
COX	Cyclooxygenase
TXA_2	Thromboxane A_2
12-HETE	12-Hydroxy-eicosatetraenoic acid
CSF	Human cerebrospinal fluid

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Introduction

Arachidonic acid (AA) and prostaglandins (PGs) are important second messengers in the central nervous system that participate in signal transduction (Connor et al. 2001), inflammation (Saeed et al. 1975, 1980) and other vital processes. Their release, turnover, and metabolism represent the 'AA cascade'. Cyclooxygenase (COX) is known to catalyze the synthesis of prostanoids after liberation of AA, an important biochemical sequela of cerebral ischemia that aggravates brain injury (Sublette et al. 2004; Sairanen et al. 1998). Thromboxane A_2 (TXA_2) formed in damaged brain tissue and after thromboembolism and subarachnoid haemorrhage is responsible for cerebral vasospasm. In addition, abnormally high levels of AA metabolites have been found in human cerebrospinal fluid (CSF) following cerebrovascular accidents (Suzuki et al. 1983).

These observations suggest that the body might control the levels of AA metabolites, so that they do not rise excessively or needlessly, and so that their elevation is not unduly prolonged. One method of control of AA metabolism would be through endogenous inhibitors of AA metabolism. Such inhibitors are found in plasma and serum from several species (Saeed et al. 1975, 1980) and might conceivably be present in CSF, where they could play an important defensive role in various pathological states. We therefore, sought to evaluate the potential of CSF to inhibit AA metabolism by blood platelets, which provide a convenient tool for studying the capacity of a substance to interfere with several pathways of AA metabolism.

Materials and methods

AA (Type 1), nordihydroguaiaretic acid (NDGA), trypsin, chymotrypsin, pronase, DNase and trypsin inhibitor were purchased from the Sigma Chemical Co., Poole, UK [^{14}C] AA (specific activity 58.4 mCi mmol^{-1}), 5,6,7,8,9,10,11, 12,14,15 (n)-[^{3}H] TXA_2 (>120 Ci mmol^{-1}) and 12 [^{3}H] hydroxy-eicosatetraenoic acid (HETE) (specific activity > 100 Ci mmol^{-1}) were obtained from Amersham PLC (Amersham, UK). Ultrafilters (Diaflo, PM10) were supplied by Amicon, Danvers, MA, USA, dialysis membrane (cut-off 12-14,000 Da) by Spectrum Los Angeles, CA, USA, and silica gel G tlc plates by Analtech, Delaware, USA.

CSF specimens were drawn by lumbar puncture from The Aga Khan University Hospital,

Karachi, Pakistan. The informed "consent" from participants enrolled in this study was obtained and samples were used fresh once they were judged to be normal after routine examination.

Human platelets from donors were routinely obtained in plastic bags containing 30–40 ml of platelet rich plasma (PRP) from the Dr. Panjwani Center for Molecular Medicine and Drug Research, ICCBS. The platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7.4) containing sodium chloride (0.15 M) and EDTA (0.2 mM). After centrifugation platelets were resuspended in the same buffer without EDTA at the initial PRP cell concentration. The PRP suspension was homogenized at 4°C using a polytron homogenizer for 15 s and the homogenate centrifuged at 1,200*g* for 20 min.

300 μL of the supernatant (containing 0.4 mg of protein) was incubated with 10 μg unlabelled AA and 0.1 μCi [1-^{14}C] AA in the presence and absence of CSF. After 15 min with gentle shaking at 37°C the reaction was stopped by adding 0.4 ml of citric acid (0.4 M) and 7 ml of ethyl acetate, and vortexed for 2 min. After centrifuging at 600*g* for 5 min at 4°C, the organic layer was separated and evaporated under nitrogen. Residues were dissolved in 50 μL ethanol and 20 μL was applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware, USA). The AA, TXB_2 (stable degradation product of TXA_2) and 12-HETE standards were spotted separately. The plates were developed in ether/petroleum ether (boiling range 40–60°C) acetic acid (50:50:1 by volume) to a distance of 17 cm. By use of this solvent system the various lipoxygenase products (HETEs) are separated with TXB_2 and PGs remaining at the origin. The solvent system used for the separation of various PGs and TXB_2 in dried organic extracts of platelet incubates as above, was ethyl-acetate: isooctane: water: acetic acid (11:5:10:2, *v*/*v*, upper phase). Radioactive zones were located and quantified by use of a Berthold T.L.C. linear analyzer and chromatography data system (Model LB 511, Berthold, Germany). All experiments were conducted with appropriate controls. The platelet (1,200*g* supernatant) boiled for 10 min, cooled and incubated with [1-^{14}C] AA using assay conditions as described above. Inactivation of the platelet enzymes by this treatment did not produce any AA metabolites (lipoxygenase product 1 (LP_1), 12-HETE or TXA_2). The radiochromatograms were compared in the absence or presence of different concentrations of CSF (as shown in the figures). The stock CSF concentration was taken as 100%. Different concentrations of this CSF were added to obtain final assay concentrations of 10, 30 and 60% CSF. The final assay volume was 1 ml.

Similarly, incubation of CSF alone with [1-^{14}C] AA in the absence of platelets (1,200*g* supernatant) also did not result in the production of AA metabolites due to possible non-enzymatic autoxidation.

Differences between control and test measurements were assessed by Student's *t* test, $P < 0.05$ was considered significant.

Result and discussion

Incubation of [1-^{14}C] AA with the homogenate (1,200*g* supernatant fraction) of human platelets resulted in the formation of two lipoxygenase products, as shown in Fig. 1 (assay volume). The relative mobility R_f (that is migration from the origin to solvent used in the presence assay system) was measured. The mobility of one product (R_f 0.62) on a silica gel G chromatogram was similar to that of that of the authentic 12-HETE. Formation of a more polar lipoxygenase product designated as LP_1 (R_f 0.1) was also observed. The absolute identification of LP_1 synthesised by platelets has been proposed to be tri-HETE, it's not confirmed or absolute. (Saeed et al. 1983, 1997).

We confirmed that tri-HETE might be the next likely product in the pathway of AA metabolism that might be formed and accumulate. The extra hydroxyl groups would then be consistent with running with a lower R_f value.

Synthesis of lipoxygenase products (LP_1 and 12-HETE) by human platelets were completely inhibited by (10–60%) human CSF. This was further confirmed by incubating human platelets with AA and NDGA a known inhibitor of lipoxygenase.

Synthesis of (LP_1 and 12-HETE) by human platelets was inhibited by human CSF (10–60%) and NDGA (nordihydroguairetic acid), a specific known inhibitor of lipoxygenase (which completely inhibited the lipoxygenation of arachidonic acid), in a concentrated-related manner (Fig. 1). Addition of NDGA dissolved in 1% ethanol completely inhibited the synthesis of lipoxygenase products (LP1 and 12-HETE). At this concentration

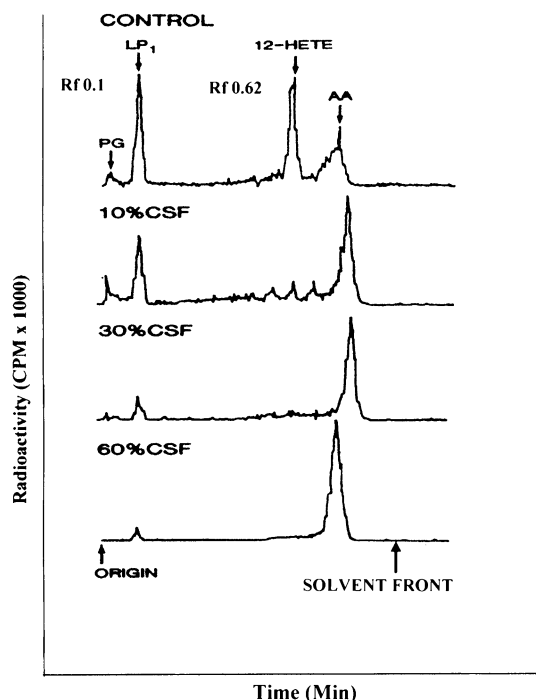

Fig. 1 Radiochromatograms scan showing the profile of arachidonate lipoxygenase metabolites produced by homogenate (1,200*g* supernatant) of human platelets with [1-14C] AA in the absence and presence of human CSF, *LP1* lipoxygenase product-1, *12-HETE* 12-hydroxyeicostatraenoic acid, *AA* arachidonic acid

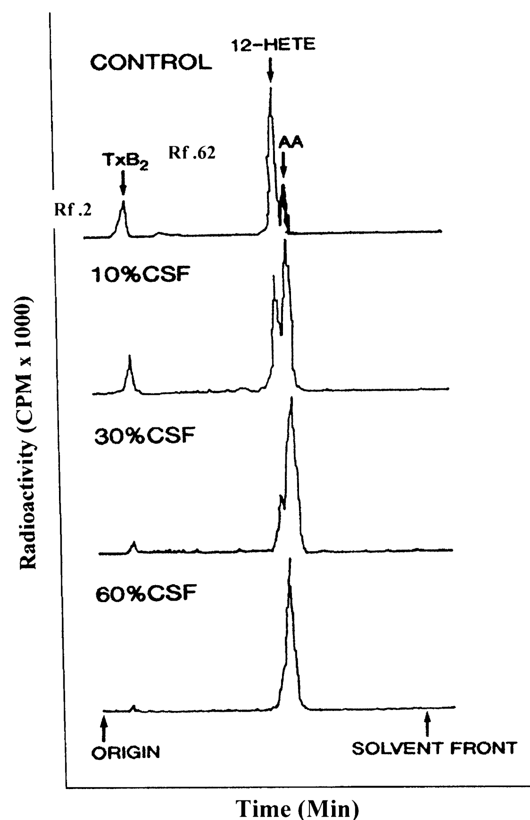

Fig. 2 Radiochromatograms of the products of cyclooxygenase metabolites of AA formed upon incubation of homogenate (1,200*g* supernatant) of human platelets with [1-14C] AA in the absence and presence of human CSF thromboxane, *AA* arachidonic acid

addition of 1% ethanol had no effect on the platelet lipoxygenase.

In similar experiments, [1-^{14}C] AA was also metabolized through platelet cyclooxygenase (COX) pathway, resulting in the formation of TXB_2 (Fig. 2) with an R_f 0.2 was identified by co-migration of TXB_2 standards.

A total of 17 CSF samples were taken from volunteers subjects. Due to unavailabilities of female volunteers no attempts were made to use female samples. The age range was 20–36 years (mean age 28 years). The inhibition of 12-HETE and LP1 formation were significantly decreased as compared to control in all 17 samples. The inhibitory effects were consistent and significant ($P < 0.05$) as there, no peak heights to be measured in the presence of CSF.

The production of TXB_2 was inhibited by CSF (10–60%) in a concentration-related manner. These results show that CSF is a dual inhibitor of platelet lipoxygenase and COX enzymes. The data were reproducible in 17 independent experiments. There were no replicates in any one experiment.

To eliminate the possibility of artifact due to binding of AA metabolites by CSF, we conducted the following experiments: 0.3 μCi each of ^{3}H-TXB_2 and 12 [^{3}H] HETE were incubated with platelet (1,200*g* supernatant) and human CSF (60% *v*/*v*) for 15 min. The reaction was stopped with citric acid. TXB_2 and 12-HETE were extracted, separated and quantified as above. Percentage losses of tritiated TXB_2 and 12-HETE that arose from non-specific binding via incubation with CSF were 5 ± 2 and 12 ± 3 ($n = 4$) respectively. In control experiments as to eliminate the possibility of artefact due to non-specific binding of different labeled (H^3) thromboxane B_2 (TXB_2) and 12 HETE. We demonstrated that the inhibition of platelet AA metabolism into TXB_2 and 12-HETE was real and not a non-specific binding of labeled TXB_2 and 12-HETE. 0.6 ml (maximum dose i.e. 60%) was incubated with labelled AA-metabolites (TXB_2 and 12-HETE). The reaction was stopped with citric acid (0.4 M) and TXB_2 and 12-HETE extracted with ethyl acetate and quantified by liquid scintillation spectrometry. Percentage losses of tritiated TXB_2 and 12-HETE were 5 ± 2 and 12 ± 3 ($n = 4$) mean (as mentioned above).

In order to gain a better understanding of the physical nature of CSF inhibitory factor(s), we subjected CSF to a number of treatments. The inhibitory activity was retained fully in the aqueous methanolic phase after extraction of CSF with a chloroform/methanol (2:1% *v*/*v*) mixture, and was stable both to heating at 100°C for 10 min, to repeated freezing and thawing, and lyophilization ($n = 7$).

Preliminary purification

Preliminary purification was done using Sephadex G-25 and membrane partition chromatography. CSF was freeze dried and dissolved in distilled water (20 mg/ml) and filtered through a Diaflo membrane PM 10 (M_r < 10,000). The filtrate, after lyophilization, was taken up in saline at a concentration of 50 mg/ml and subjected to chromatographic separation on columns of Sephadex G-25. The human CSF produced only one peak of UV absorbance at 280 mm. The fraction was eluted with saline and tested for COX and LOP inhibitory effects, which coincided with one peak eluting at a molecular weight of about 1,400).

Nature of inhibitor

Before the assay for inhibitory activity human CSF was preincubated for 17 h at 37°C, with gentle shaking, with trypsin (EC 3.4.21.4) from bovine pancreas, twice crystallized (Sigma), or with α-chymotrypsin (EC 3.4.21.1) from bovine pancreas, thrice crystallized (Sigma), or with DNase I (EC 3.1.21.1) from porcine spleen, or DNase II Type IV (Sigma).

After incubation, the action of trypsin or α-chymotrypsin was stopped by adding trypsin inhibitor from bovine pancreas (Sigma). The action of DNase I and II was stopped by boiling for 5 min. Preincubation with trypsin or α-chymotrypsin at 0.5, 5 or 50 μg/ml completely abolished the inhibitory activity of CSF, whereas preincubation with DNase I or II at these concentrations had no effect. Trypsin, α-chymotrypsin, DNase I or DNase II alone did not affect the inhibitory activity of CSF at the concentrations used in the assay.

It can be argued that the inhibition of AA metabolism by CSF might be due to the presence of catalytic enzymes trypsin or α-chymotrypsin, DNase I or DNase II. In order to rule out this possibility, a control experiment was performed using trypsin or α-chymotrypsin, DNase I or DNase II alone. It was observed that trypsin or α-chymotrypsin, DNase or DNase II alone did not affect the platelet AA metabolizing enzymes at the concentrations used in the assay.

The CSF inhibitory activity was stable to boiling for 10 min or incubation at 37°C for 20 h, repeated freezing and thawing also did not affect the inhibitory activity. These experiments indicate that the inhibitor is peptidic in nature and not associated with DNA.

Enzyme sensitivity studies: CSF was pre-incubated for 17 h at 30°C, with trypsin, α-chymotrypsin, DNase I (Type I), or DNase II (Type IV). The action of trypsin or α-chymotrypsin was then stopped by adding trypsin inhibitor (bovine pancreas), or the action of DNase I and II was stopped by boiling for 5 min.

Pre-incubation with trypsin or α-chymotrypsin at 0.5, 5 or 50 μg ml^{-1} completely abolished the inhibitory activity of CSF, whereas pre-incubation with DNase I DNase II at these concentrations had no effect. Trypsin, α-chymotrypsin, DNase I or DNase II alone did not affect the platelet AA metabolizing enzymes at the concentrations used in the assay. These experiments indicate that the inhibitor is peptidic in nature and not associated with DNA.

Inhibitory activity eluted at one bed volume [on a Sephadex G-25 column (1.5 × 90 cm)] previously equilibrated with saline. The fractions were eluted with saline for 20 min at a flow rate of 33 ml h^{-1}. in aliquots (0.2 ml) of each fraction (volume: 11 m), inhibitory activity on platelet AA metabolising was estimated. The results suggested that the molecular weight of CSF inhibitory peptide was around 1,400 Da. Our result strongly suggests that this peptide is responsible for the inhibition of platelet cyclooxygenase (COX) and lipoxygenase.

In additional experiments, CSF inhibitory peptide completely passed through (Amicon) PM-10 filter, and was not detectable in CSF that had been subjected to membrane dialysis against 0.15 M NaCI for 48 h at 4°C (n = 4).

These results show a hitherto unrecognized property of human CSF. To our knowledge inhibitors of AA metabolism in CSF have not been described previously, and our observations indicate a biochemical nature distinctly different for the endogenous inhibitors of AA metabolism previously found in human plasmas, serum or various tissue cytosols, all of which are macromolecular in nature (Saeed et al. 1982, 2003; Saeed and Ahmed 2006; Coffey et al. 2004). Taken together, the present and previous findings are indicative of the presence of quite distinct kinds of endogenous inhibitors of AA metabolism in CSF as compared with plasma. It seems possible that such an inhibitor of AA metabolism, in CSF, might play a physiological role in the regulation of AA metabolism in CSF, in the sub-arachnoid space, and the adjacent tissues.

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References

Coffey MJ, Jarvis GE, Gibbins JM, Coles B, Barrett NE, Wylie OR, O'Donnell VB (2004) Platelet 12-lipoxygenase activation via glycoprotein VI: involvement of multiple signaling pathways in agonist control of H(P)ETE synthesis. Circ Res 94:1598–605

Connor JD, Rasheed H, Gilani AH, Cheema M, Rizvi Z, Saeed SA (2001) Second messengers in platelet aggregation evoked by serotonin and A23187, a calcium ionophore. Life Sci 69:2759–2764

Saeed SA, Ahmed S (2006) Role of cyclooxygenase-2 in myocardial infarction and ischaemia. J Coll Physicians Surg Pak 16:324–328
Saeed SA, Cuthbert J (1975) On the mode of action and biochemical properties of anti-inflammatory drugs-II. Prostaglandins 13:565–576
Saeed SA, Mitchell MD (1982) Stimulants of prostaglandin biosynthesis in human fetal membranes, uterine decidua vera and placenta. Prostaglandins 24:475–484
Saeed SA, Mitchell MD (1983) Conversion of arachidonic acid to lipoxygenase products by human fetal tissues. Biochem Medic 30:322–327
Saeed SA, McDonald-Gibson WJ, Cuthbert J, Copas JL, Schneider C, Gardiner PJ et al (1975) Endogenous inhibitor of prostaglandin synthetase. Nature 270:32–36
Saeed SA, Drew M, Collier HOJ (1980) Endogenous inhibitors of lipoxygenase. Eur J Pharmacol 67:169–170
Saeed SA, Gilani AH, Majoo RU, Shah BH (1997) Anti-thrombotic and anti-inflammatory activities of protopine. Pharmacol Res 36:1–7
Saeed SA, Khan SK, Samnani SAA, Farhat K, Hansraj NI, Khan AMA et al (2003) The inhibition of prostaglandin biosynthesis by human plasma and its relationship to albumin and haptoglobin. J Biol Sci 3:1188–1202
Sairanen T, Ristimaki A, Karjalainen-Lindsberg ML, Paetau A, Kaste M, Lindsberg PJ (1998) Cyclooxygenase-2 is induced globally in infarcted human brain. Ann Neurol 43:738–747
Sublette ME, Russ MJ, Smith GS (2004) Evidence for a role of the arachidonic acid cascade in affective disorders: a review. Bipolar Disord 6:95–105
Suzuki N, Nakamura T, Imabayashi S, Ishikawa Y, Sasaki T, Asano T (1983) Identification of 5-hydroxy eicosatetroaenoic acid in cerebrospinal fluid after subarachnoid hemorrhage. J Neurochem 41:1186–1189

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