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PHOSPHOLIPASE INHIBITION AND THE MECHANISM OF ANGIOTENSIN-INDUCED PROSTACYCLIN RELEASE FROM RAT MESENTERIC VASCULATURE

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Abstract—Generation of prostaglandins by arterial vasculature of rats was measured by perfusing the isolated mesenteric arterial vascular bed with Krebs–Henseleit solution. The effluent directly superfused bioassay tissues in cascade, and aliquots were collected for subsequent chromatography and radioimmunoassay. Injection of arachidonate (1–10 μg) or angiotensin II (0.1–0.5 μg) through the mesentery caused release of a PGI_2-like substance. After extraction and chromatographic separation of the mesenteric effluent, it was confirmed by radioimmunoassay that 6-oxo-$PGF_{1\alpha}$ (the hydration production of PGI_2) is the predominant prostanoid generated from exogenous arachidonate. Release of 6-oxo $PGF_{1\alpha}$ and PGE_2 from mesentery was also stimulated by injection of angiotensin II (0.05–0.5 μg). Treatment of the mesentery with indomethacin (1 μg/ml) abolished all effects of angiotensin II and arachidonate. Perfusion of the mesentery with dexamethasone (3 μg/ml) or mepacrine (33 μg/ml) both of which have been reported to inhibit phospholipase A_2 activity, reduced PGI_2 release induced by angiotensin II, but did not affect conversion of exogenous arachidonate. It is concluded that PGI_2 is the major prostanoid generated in perfused mesenteric arterial vasculature of rats, and angiotensin II releases PGI_2 by activation of a phospholipase.

INTRODUCTION

Prostacyclin (PGI_2), a labile metabolite of arachidonic acid, which relaxes vascular smooth muscle [1, 2] and inhibits platelet aggregation [3], is produced by isolated blood vessels [3, 4]. Local release of prostaglandins in isolated vascular tissue is thought to be involved in regulation of the vasoconstrictor actions of angiotensin II and the vasodilator actions of bradykinin [5–8]. Bioassay and chromatographic techniques were used to identify these prostaglandins as PGE-like [5, 7]. However, these methods did not readily distinguish between PGE_2 and PGI_2 or 6-oxo-$PGF_{1\alpha}$, the stable degradation product of PGI_2. Improved bioassay [9], radioimmunoassay [10, 11] and chromatographic [12–14] methods have been developed which have enabled the identification of PGI_2 as the major metabolite of arachidonic acid synthesized by blood vessels [3].

The present work combines pharmacological and biochemical methods to investigate the regulation by angiotensin II of arachidonic acid metabolism in isolated mesenteric vasculature.

MATERIALS AND METHODS

Preparation of rat isolated mesenteric vasculature. Male Sprague–Dawley rats, weight range 250–350 g, were anaesthetized with ether and the superior mesenteric artery cannulated 1 cm distal to the aorta. The vessels of the mesenteric arterial bed were severed close to the intestine and perfused at 3 ml/min with Krebs–Henseleit solution gassed with 5 per cent CO_2 in oyxgen, as described by McGregor [15].

The mesenteric effluent superfused a cascade of bioassay tissues [16].

Bioassay technique. Prostanoids in the effluent from the mesenteric vasculature were detected by bioassay tissues: bovine coronary artery, rat stomach strip and rat colon, which distinguish PGE_2, $PGF_{2\alpha}$, PGI_2 and TxA_2 [2, 9, 17]. The bioassay tissues were treated with indomethacin (1 μg/ml) and the antagonist, Sar^1 Ile^8-angiotensin II (0.03 μg/ml), to prevent intramural generation of prostanoids by the tissues and to render them insensitive to angiotensin II. Changes in length of the bioassay tissues were measured with auxotonic levers attached to Harvard smooth muscle transducers and recorded on a Rikadenki recorder, or Grass (Model 7) polygraph.

Chromatography of arachidonic acid metabolites. In order to characterize precisely the arachidonic acid metabolites produced, 3 ml of mesenteric effluent were collected during perfusion under resting conditions, after injection of angiotensin II (0.5 μg), and after injection of arachidonate (5 μg) into the perfusion medium. The effluent was acidified to pH 3.5 with formic acid and extracted with 3 vol. of ethyl acetate. The organic layer was evaporated under nitrogen and resuspended in 0.5 ml of column solvent. For high-pressure liquid chromatography, the Waters Associates instrument was used with a 30 cm × 3.9 mm i.d. reversed-phase fatty acid analysis column (Waters Associates). The separation

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† Abbreviations: PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostacyclin; 6-oxo-$PGF_{1\alpha}$, 6-oxo prostaglandin $E_{1\alpha}$; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2.

method used was based on that of Alam *et al.* [14]. The arachidonic acid metabolites were eluted isocratically with a solvent system consisting of acetonitrile–water–benzene–acetic acid (230:767:2:1). The flow rate was 2 ml/min. Fractions of 2 ml were collected, lyophilized and assayed by radioimmunoassay for 6-oxo-$PGF_{1\alpha}$, TxB_2, $PGF_{2\alpha}$ and PGE_2. Recoveries of added [^{3}H] PGE_2, [^{3}H] $PGF_{2\alpha}$, [^{3}H] 6-oxo-$PGF_{1\alpha}$ and [^{3}H] TxB_2 were monitored and data were corrected for the average loss. In all other experiments prostanoid measurements were obtained by direct radioimmunoassay of the effluent samples without extraction and chromatography.

Radioimmunoassay of prostanoids. Rabbit antisera, directed against 6-oxo-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and TxB_2 were diluted (6-oxo-$PGF_{1\alpha}$, 1:6,000; PGE_2, 1:10,000; $PGF_{2\alpha}$, 1:30,000; TxB_2 IgG, 0.5 μg/0.3 ml) in Tris-gelatin buffer (15 mM Tris, 140 mM NaCl, pH 7.4, gelatin 500 mg/l) to give approximately 40 per cent of total cpm bound in the absence of unlabelled ligand. Aliquots (0.1 ml) of the appropriate antibody were mixed with standard or sample (0.1 ml diluted in Tris-gelatin buffer) in plastic tubes. The range of each standard used was 8–2000 pg per tube. Samples were diluted to give a value on the linear part of the binding curve. Approximately 4000 cpm of the appropriate tritiated prostanoid were added to each tube in 0.1 ml Tris-gelatin buffer to give a total reaction volume of 0.3 ml. After overnight incubation at 4° the protein-bound material was separated by adding 0.3 ml of a suspension of dextran-coated charcoal (6 mg/ml charcoal, 1 mg/ml bovine serum albumin and 1 mg/ml dextran T70 in Tris-gelatin buffer). The tubes were vortex-mixed, incubated at 4° for 5 min and centrifuged for 10 min at 3000 rpm at 4° in a M.S.E. Coolspin centrifuge. The supernatant, containing bound ligand, was decanted into scintillation vials with 5 ml Instagel (Packard) scintillation cocktail, and the radioactivity determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. All radioimmunoassays were carried out in duplicate. Sensitivities of the assays, determined by the amount of standard per assay tube required to inhibit binding of the label to the antibody by 10 per cent, were 5 pg for 6-oxo-$PGF_{1\alpha}$, 25 pg for PGE_2, 10 pg for $PGF_{2\alpha}$ and 10 pg for TxB_2. Cross reactivities with the PGE_2 antibody were: 6-oxo-$PGF_{1\alpha}$, 0.12 per cent; $PGF_{2\alpha}$, 1 per cent; TxB_2, 0.007 per cent and arachidonic acid, 0.001 per cent. Cross reactivities with the 6-oxo-$PGF_{1\alpha}$ antibody were: PGE_2 0.5 per cent; PGE_1 0.25 per cent; TxB_2 0.0006 per cent; $PGF_{2\alpha}$ 0.1 per cent; arachidonic acid 0.0002 per cent. Pre-chromatography samples were not assayed for $PGF_{2\alpha}$ and TxB_2. Intra-assay coefficients of variation were 4.0 per cent for 6-oxo-$PGF_{1\alpha}$ and 4.1 per cent for PGE_2. Interassay coefficients of variation were 12.4 per cent for 6-oxo-$PGF_{1\alpha}$ and 18.5 per cent for PGE_2. All pre-chromatography samples were assayed by diluting the Krebs–Henseleit effluent in Tris-gelatin buffer. Nonspecific effects of the Krebs-Henseleit buffer were ruled out by addition of standard prostanoid to Krebs–Henseleit buffer and dilution in Tris-gelatin buffer. The curve obtained was parallel to the curve with prostanoid in Tris-gelatin buffer.

Materials. Prostaglandins and their metabolites were gifts from Dr. J. E. Pike, Upjohn Co. (Kalamazoo, U.S.A). PGI_2 was obtained as sodium salt. Stock solutions were made up each day in 0.01 M sodium bicarbonate buffer (pH 10) and kept on ice. PGE_2 and dilutions of PGI_2 were prepared in ice-cold 0.05 M Tris buffer (pH 7.6) immediately before use. Arachidonic acid (Sigma Grade 1) was stored in *n*-hexane (10 mg/ml) under nitrogen at −20°. Immediately before use the solvent was evaporated under nitrogen and the arachidonic acid dissolved in 0.1 M sodium carbonate making the sodium salt. Stock solution of angiotensin II (hypertensin, Ciba) were prepared in 0.05 M acetic acid and stored at −20°. Aliquots were thawed on the day of the experiment and diluted in 0.15 M sodium chloride. Mepacrine (Sigma), dexamethasone (Decadron, M.S.D.) and Sar^1-Ile^8-angiotensin II (Bachem Inc.) were dissolved in 0.15 M sodium chloride daily. Indomethacin (Sigma) was dissolved in 1 M Tris buffer (pH 8.4) each day and diluted in 0.05 M Tris buffer (pH 7.6) before use.

Antibodies used were diluted rabbit antisera directed against PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$. The antibody for thromboxane B_2 (TxB_2), the hydration product of TxA_2, was obtained as purified anti-TxB_2 rabbit IgG (a gift from Dr. J. Bryan Smith). Anti-PGE_2 antiserum was a gift from Dr. L. Levine; anti-$PGF_{2\alpha}$ antiserum (ONO Pharmaceutical Co., Japan) was a gift from Professor G. D. Thorburn; anti-6-oxo-$PGF_{1\alpha}$ antiserum was a gift from Dr. L. C. Best. ^{3}H-labelled arachidonic acid metabolites were obtained from New England Nuclear Corp. (Boston, MA) or the Radiochemical Centre (Amersham, U.K.).

RESULTS

Radioimmunoassay of prostanoids in the rat mesenteric effluent after extraction and separation showed release of PGI_2 (measured as 6-oxo-$PGF_{1\alpha}$),

Table 1. Effect of phospholipase and cyclo-oxygenase inhibitors on AII-induced output of 6-oxo$PGF_{1\alpha}$

Inhibitor	Concentration (μg/ml)	6-oxo-$PGF_{1\alpha}$* (ng/ml) No inhibitor	Inhibitor present
Indomethacin	1	1.9	0.5
Dexamethasone	3	3.4	0.5
Mepacrine	33	5.1	0.6

* Increase of 6-oxo-$PGF_{1\alpha}$ (measured by radioimmunoassay) in the mesenteric effluent 1 min after injection of AII (0.25 or 0.5 μg), both before and after inhibitor. Results from 3 representative experiments are presented.

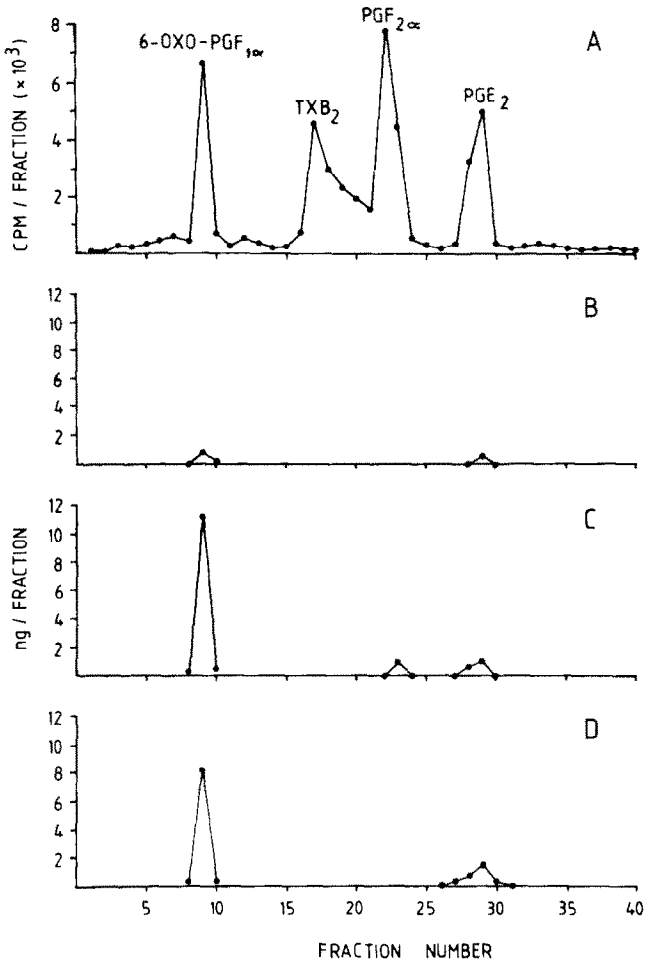

Fig. 1. High-pressure liquid chromatogram of arachidonic acid metabolites. (A) Separation of radioactive metabolites. (B) Separation of metabolites released into the effluent from the isolated perfused rat mesenteric artery. Every fraction was assayed by radioimmunoassay for each arachidonic acid metabolite and only one major peak of serologic activity was observed with each antiserum. (C) Separation of the metabolites released into the effluent after injection of exogenous arachidonate (5 μg) into the mesentery, and (D) separation of arachidonic acid metabolites released after stimulation by AII (0.5 μg).

PGE_2 and $PGF_{2\alpha}$ (Fig. 1). No TxA_2 (measured as TxB_2) release was detected. The chromatographic separation method yielded highly reproducible results, with predictable retention times for all four arachidonic acid metabolites. The shoulder to the TxB_2 peak was a consistent feature of the chromatographic behaviour of this prostanoid, since it was seen whether ^{3}H-labelled TxB_2 was used (Fig. 1) or radioimmunoassay of unlabelled TxB_2 (data not shown). 6-oxo-$PGF_{1\alpha}$ was the predominant prostanoid released by arachidonic acid and angiotensin II. It was confirmed by direct radioimmunoassay that angiotensin II increased 6-oxo-$PGF_{1\alpha}$ production to a greater extent than it did PGE_2 (Fig. 2); the ratio of 6-oxo-$PGF_{1\alpha}$ to PGE_2 was increased from 1.9 to 4.3 by angiotensin II infusion. Similar ratios were found when chromatographic separation was applied.

Bioassay of the mesenteric effluent supported the evidence that PGI_2 is the major prostanoid released from the perfused mesenteric artery with smaller amounts of other prostanoids (e.g. PGE_2) contributing to the rat stomach strip contraction (Fig. 3 and [18]). Mepacrine (33 μg/ml) caused a marked reduction of angiotensin II-induced release of the PGI_2-like activity as detected by bioassay. This effect was detected within 30 min, and reversed within 30 min of terminating the infusion. Conversion of exogenous arachidonate was unchanged (Fig. 3). During perfusion with dexamethasone (3 μg/ml) the release of the PGI_2-like substance induced by angiotensin II (0.1–0.5 μg) was progressively reduced, and abolished after 1–3 hr without affecting conversion of exogenous arachidonate (results not shown).

DISCUSSION

The value of defining a tissue's pattern of arachidonic acid metabolism by high pressure liquid chromatographic separation of extracts followed by

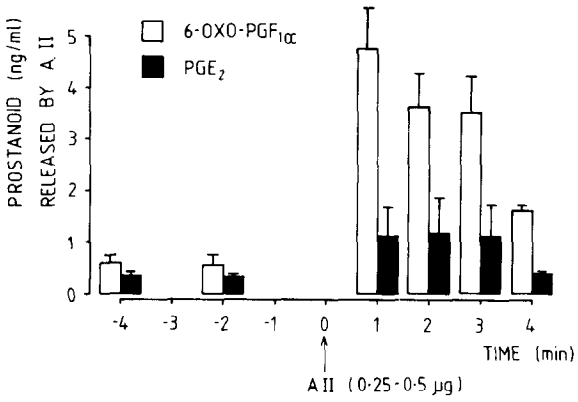

Fig. 2. Time course of 6-oxo-$PGF_{1\alpha}$ and PGE_2 production after stimulation by AII. The levels of 6-oxo-$PGF_{1\alpha}$ and PGE_2 at the time indicated were measured by radioimmunoassay. Data are mean ± S.E.M. from 4–9 experiments. The increase in 6-oxo-$PGF_{1\alpha}$ is highly significant ($P < 0.001$), whereas the increase in PGE_2 is not significant (unpaired Student's *t*-test).

radioimmunoassay has been pointed out by Levine and his colleagues [14, 19]. In the present work this approach has been combined with biological assay data to confirm that PGI_2 is the major prostanoid produced by the perfused rat mesenteric vasculature, both when stimulated by angiotensin II and when exogenous arachidonic acid is provided [18, 20]. Having defined the products specifically in this way under given experimental circumstances, and knowing the cross reactivities of the various antisera used in the radioimmunoassays, it is possible to proceed with direct chemical assays of perfusates in parallel with biological assays.

Indomethacin inhibited the increase in prostanoid generation induced either by angiotensin II or by exogenous arachidonate. The fact that dexamethasone and mepacrine inhibited angiotensin-induced, but not arachidonate-induced release of prostanoids suggests that the primary action of angiotensin is to mobilize arachidonic acid from its esterified form in membrane phospholipid. In this respect the action of angiotensin resembles that of bradykinin [21, 22], which is used as a classical phospholipase A_2 stimulator. However, it should be noted that both mepacrine and corticosteroids failed to block liberation by bradykinin of [^{14}C]oleic acid from previously labelled phospholipid stores in isolated lungs [23].

The mechanisms of arachidonate liberation are currently the subject of some controversy. Until recently phospholipase A_2 was thought to be responsible for release of arachidonate, and inhibition of prostaglandin release by dexamethasone and mepacrine was explained by inhibition of phospholipase A_2 activation [23–25]. On the basis of evidence obtained first in the platelet [26] and subsequently in cultured cells [27] it is now proposed that two distinct enzymic steps are involved in the hydrolysis of membrane phospholipid which results in availability of arachidonic acid for further metabolism. These two enzymes are a phosphatidyl inositol specific phospholipase *c* and a diglyceride lipase [26, 28] or a diglyceride kinase [29]. The effects of anti-inflammatory steroids or mepacrine on these enzymes have not been studied. Therefore the possibility must also be considered that angiotensin II acts through phospholipase C in blood vessels, particularly since bradykinin has been shown to activate this enzyme in mouse fibrosarcoma cells [27].

We [18, 30] and others [8, 20] have noted that angiotensin II releases a PGI_2-like substance from lung, kidney and other vascular organs, but does not activate prostanoid biosynthesis in extravasated

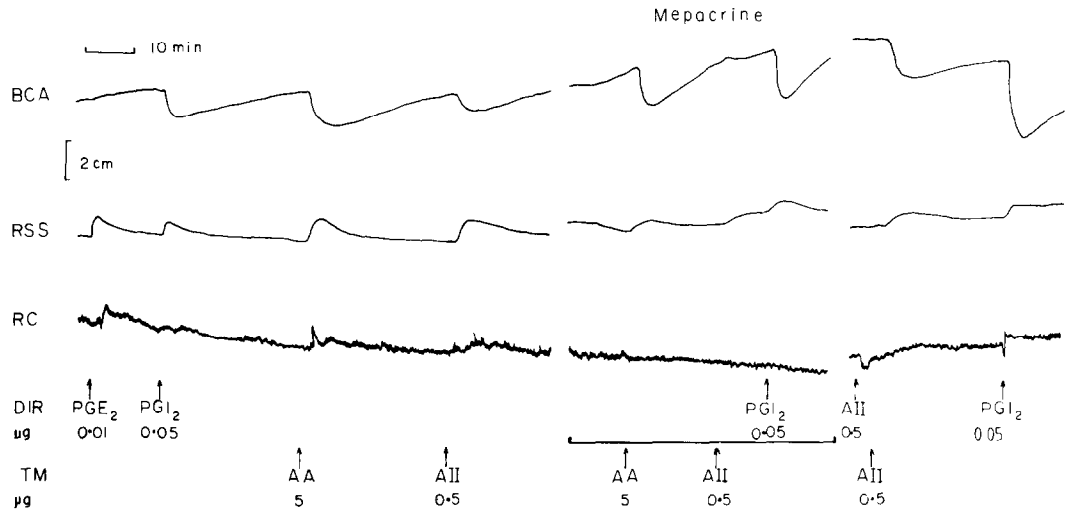

Fig. 3. The effect of mepacrine on arachidonate (AA)- and angiotensin (AII)- induced release of prostanoids from rat mesenteric vasculature. Bovine coronary artery (BCA), rat stomach strip (RSS) and rat colon (RC) are continuously superfused with the mesenteric effluent. Injection of arachidonate or angiotensin II through the mesentery (TM) causes responses of BCA and RSS which are mimicked by PGI_2 added directly (DIR) to the bioassay tissues. In the centre panel mepacrine (33 μg/ml) is perfused through the mesentery.

blood [8, 30] which does convert exogenous arachidonic acid into TxA_2 and prostaglandins [31]. Furthermore, the release of prostanoids involves an angiotensin II receptor, since it is abolished by the selective antagonists Sar^1-Ile^8- and Sar^1-Ala^8-angiotensin II [18, 20, 30]. The receptor must therefore be linked to a phospholipase in lungs and vascular tissue but is either not present or is not linked to phospholipase in the cellular components of extravasated blood. The factors which determine the predominant production of PGI_2 from liberated arachidonic acid in vascular tissue remain to be clarified.

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