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Recovery of prostacyclin synthesis by rabbit aortic endothelium and other tissues after inhibition by aspirin

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1 The effect of aspirin on prostacyclin (PGI_2) and thromboxane B_2 (TXB_2) synthesis was studied in rabbits. Tissues were removed from animals killed at intervals after injection of aspirin, and incubated with Hanks' solution. PGI_2 synthesis was monitored by radioimmunoassay of its hydrolysis product, 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$). TXB_2 production in clotted blood, also measured by radioimmunoassay, was determined as an index of platelet cyclo-oxygenase activity.

2 6-oxo-$PGF_{1\alpha}$ and TXB_2 production 0.5 h after aspirin were similarly inhibited to less than 5% of control in all incubations. Subsequent recovery of PGI_2 synthesis occurred more rapidly in aortic endothelium than in other tissues, including aorta denuded of endothelium. Recovery of TXB_2 production was slower than that of PGI_2.

3 Intravenous cycloheximide prevented the partial recovery of PGI_2 synthesis that otherwise occurred 6 h after aspirin, while intravenous epidermal growth factor increased recovery.

4 It is concluded that in the rabbit, cyclo-oxygenase is synthesized more rapidly in aortic endothelium than in deeper layers of aorta, or in the other tissues studied.

Introduction

Aspirin inhibits prostaglandin synthesis (Vane, 1971; Smith & Willis, 1971; Ferreira *et al.*, 1971) by irreversible acetylation of fatty acid cyclo-oxygenase (Roth & Majerus, 1975). However, cultured cells recover the ability to synthesize prostacyclin (PGI_2) after inhibition by aspirin as a result of synthesis of cyclo-oxygenase (Jaffe & Weksler, 1979; Czervionke *et al.*, 1979). The rate of recovery depends critically on culture conditions (Dejana *et al.*, 1983). *In vivo* studies are therefore needed to determine the rate of cyclo-oxygenase regeneration in life. However, such studies have yielded seemingly conflicting information. PGI_2 synthesis by human venous tissue excised at intervals following aspirin ingestion remains inhibited 48 h after the dose (Hanley *et al.*, 1981). In contrast, bradykinin-stimulated PGI_2 synthesis *in vivo* recovers from inhibition by aspirin in 6 h (Heavey *et al.*, 1985). PGI_2 synthesized by chopped blood vessels is derived mainly from vascular media (Moncada *et al.*, 1977), while it is possible that bradykinin-stimulated PGI_2 originates from endothelium. To explain the different rates of recovery after aspirin observed by Hanley *et al.* (1981) and Heavey *et al.* (1985), we therefore proposed that cyclo-oxygenase is synthesized more rapidly in endothelium than in deeper vascular layers (Heavey *et al.*, 1985).

The object of the present experiments was to test this hypothesis. Experiments were performed on rabbits, using a method of measuring endothelial PGI_2 synthesis in which a portion of aorta is mounted in a device with chambers containing Hanks' solution directly overlying the endothelium (Eldor *et al.*, 1981). The ability of aortic endothelium to synthesize PGI_2 after inhibition by aspirin was compared with PGI_2 synthesis by chopped aorta denuded of endothelium and other tissues. Thromboxane B_2 (TXB_2) was measured in serum from blood allowed to clot at 37°C (serum TXB_2), as an index of platelet cyclo-oxygenase activity. Platelets do not make protein, and recovery of serum TXB_2 after aspirin probably reflects enzyme synthesis in megakaryocytes (Walenga *et al.*, 1984), with subsequent entry of young platelets into the circulation (Patrono *et al.*, 1980). Some animals were treated with cycloheximide, an inhibitor of protein synthesis (Trakatellis *et al.*, 1965), or with epidermal growth factor (EGF), which increases synthesis of cyclo-oxygenase *in vitro* (Bailey *et al.*, 1985). The object was to determine if these compounds, administered *in vivo*, influence the recovery of PGI_2 synthesis after inhibition by aspirin.

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Methods

Aspirin (Sigma) 10 mg kg^{-1} was injected into the marginal ear veins of male New Zealand White rabbits (2.4–3.5 kg, $n = 6$ for each experiment). Aspirin (80 mg ml^{-1}) was dissolved immediately before use in 1 M Tris buffer (pH 8.4) at room temperature, and diluted with 0.15 M sodium chloride to 10 mg ml^{-1} (Amezcua *et al.*, 1978). Control animals were injected with this vehicle alone. EGF (mouse submaxillary gland, Sigma) was dissolved in 0.15 M sodium chloride, and injected, 2 $\mu g\,kg^{-1}$ i.v. at 3 h, and 1 $\mu g\,kg^{-1}$ i.v. at 4 h, after aspirin or vehicle. Cycloheximide (Sigma), was prepared as a slurry by adding 2 g of solid to 4 ml of ethanol, and dissolved with 0.15 M sodium chloride (4 ml) at room temperature. Cycloheximide (100 mg kg^{-1} i.v.) was injected immediately before aspirin or vehicle and again (50 mg kg^{-1} i.v.) at 2 h and 4 h.

Animals were killed by an intravenous injection of pentobarbitone. The thoracic aorta, pericardium, a portion of peritoneum from the posterior abdominal wall, a segment of ileum, the stomach and the heart were removed rapidly and placed in Hanks' solution (Gibco U.K.) at 4°C. A segment of left ventricular free wall was dissected, a rectangle of gastric mucosa separated from the underlying smooth muscle, and the ileal lumen flushed with Hanks' solution. Tissues were chopped into 1 mm cubes using a McIlwain tissue chopper. The aorta was cut into two lengths. One was stripped of endothelium by rubbing with filter paper (Furchgott & Zawadzki, 1980) and chopped as for the other tissues. The other was divided longitudinally between the origins of the intercostal arteries, opened and placed, endothelial surface uppermost, on a filter paper moistened with Hanks' solution, on the perspex base of a template chamber (Eldor *et al.*, 1981). The chamber was made in the workshop of the Royal Postgraduate Medical School. Its upper part was held in place by spring mounted screws and contained four wells (0.7 cm diameter) directly overlying the endothelium. Hanks' solution (0.25 ml) at 37°C was added to each well, temperature being maintained by a heating lamp. The preparation was agitated gently for 5 s each minute. Chopped tissues were incubated at 37°C in Hanks' solution (1 ml) with constant shaking. Incubations were of 15 min duration and were terminated by aspiration of the medium, which was stored at −20°C for subsequent assay.

Radioimmunoassay was performed in triplicate on unextracted samples using approximately 5 nCi of [^{3}H]-6-oxo-$PGF_{1\alpha}$ (Amersham U.K.) per tube and a final dilution of antiserum of 1:15,000. Antibody to 6-oxo-$PGF_{1\alpha}$ (Hensby *et al.*, 1981; Orchard *et al.*, 1982), was a gift from Dr L. Myatt (Institute of Obstetrics, Hammersmith Hospital, London). Unbound ligand was separated with activated charcoal; 50% displacement of tritiated ligand was caused by 61.5 ± 2.9 pg (mean ± s.e. mean) of standard 6-oxo-$PGF_{1\alpha}$ (Upjohn, Kalamazoo, Michigan, U.S.A.). Inter-assay and intra-assay variation were 14% and 7% respectively. Most samples were diluted with phosphate buffered gelatin saline so that 0.1 ml caused 20–80% displacement of [^{3}H]-6-oxo-$PGF_{1\alpha}$, and non-specific interference excluded by performing the triplicate assays at different dilutions. Incubations of tissue from animals treated with aspirin 0.5 h before death, and from animals treated with aspirin and cycloheximide, contained only small amounts of 6-oxo-$PGF_{1\alpha}$, and assays were performed on 0.4 ml portions of undiluted sample.

Before injecting animals with aspirin or vehicle, and again immediately before killing them, 2 ml of blood was drawn from an ear vein with a 21 gauge Butterfly needle (Abbott, Sligo, Republic of Ireland), and placed in a plain glass tube at 37°C for 1 h (Patrono *et al.*, 1980). The resulting serum was separated from clot by centrifugation at 1000 *g* for 10 min at 4°C. The concentration of TXB_2 in serum was measured by a procedure similar to that used for 6-oxo-$PGF_{1\alpha}$ determination apart from the use of approximately 15 nCi of [^{3}H]-TXB_2 (Amersham U.K.) per tube and a final dilution of antiserum of 1:50,000 of an antibody of TXB_2. The properties of this antibody have been described previously (Burch *et al.*, 1979). It was obtained as a gift from Dr P. Halushka (Department of Pharmacology, University of South Carolina, Charleston, U.S.A.).

Analysis

Results are expressed as percentages of control value. Means are given ± s.e. mean. Differences were evaluated by the rank sum test, and considered significant when $P < 0.05$.

Results

The effect of aspirin (10 mg kg^{-1}, i.v.) is shown in Table 1. At 0.5 h after aspirin prostanoid production by tissues and blood was almost abolished: in no individual incubation was 6-oxo-$PGF_{1\alpha}$ production greater than 5% of the mean control value (Table 1), and the mean inhibition was greater than 98% in all tissues. At 6 h, production of TXB_2 in clotted blood remained almost completely inhibited (0.35 ± 0.12% control), but there was partial recovery of 6-oxo-$PGF_{1\alpha}$ synthesis by all tissues. Recovery was greatest in aortic endothelium (24 ± 7% control), significantly more than that by chopped aorta denuded of endothelium (4.2 ± 0.9% control) or other tissues (range: 2–11% control). At 24 h the concentration of TXB_2 in serum was 5.48 ± 1.36% control, reflecting significan-

Table 1 Inhibition of tissue 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) production and of serum thromboxane B_2 (TXB_2) by aspirin (10 mg kg^{-1}) in the rabbit

Tissue	*Prostanoid production[a] (% control ± s.e. mean)* *0.5 h*	*6 h*	*24 h*
Aortic endothelium	1.8 ± 0.61	23.7 ± 7.04	64.1 ± 7.65
Aortic media/adventitia	1.6 ± 0.32	4.2 ± 0.86*	32.8 ± 7.62*
Pericardium	1.1 ± 0.25	10.6 ± 4.20*	45.8 ± 11.00*
Peritoneum	1.4 ± 0.61	6.1 ± 1.64*	43.2 ± 8.40*
Ileum	1.0 ± 0.43	4.7 ± 1.62*	24.7 ± 5.88*
Gastric mucosa	0.2 ± 0.06	3.0 ± 1.06*	46.2 ± 13.09*
Cardiac ventricle	< 0.3	2.2 ± 0.67*	18.6 ± 4.41*
Serum TXB_2	0.12 ± 0.04	0.35 ± 0.12†	5.48 ± 1.36†

[a]6-oxo-$PGF_{1\alpha}$ production by tissues and TXB_2 production by blood clotted at 37°C (serum TXB_2).
*Significantly less than recovery of aortic endothelial production ($P < 0.05$).
†Significantly less than recovery of 6-oxo-$PGF_{1\alpha}$ production by all tissues ($P < 0.05$).

tly less recovery of cyclo-oxygenase activity in platelets than in the tissues that synthesize PGI_2. 6-oxo-$PGF_{1\alpha}$ production by aortic endothelium was 64.1 ± 7.65% control, significantly more than production of 6-oxo-$PGF_{1\alpha}$ by chopped aorta denuded of endothelium (32.8 ± 7.62% control), or other tissues (range: 18.6–46.2% control).

Cycloheximide (100 mg kg^{-1} i.v.) was given immediately before aspirin (100 mg kg^{-1}), or the vehicle used to dissolve aspirin, and again (50 mg kg^{-1} i.v.) 2 h and 4 h later. Animals were killed at 6 h. Cycloheximide augmented PGI_2 synthesis by each tissue in animals treated only with the vehicle, by 1.2–12.9 fold (Table 2). However, it abolished the partial recovery of PGI_2 synthesis that otherwise occurred 6 h after aspirin (Figure 1): in animals treated with cycloheximide, tissue PGI_2 production 6 h after aspirin was similar to that at 0.5 h in animals treated with aspirin alone.

EGF was given in doses of 2 μg kg^{-1} and 1 μg kg^{-1} i.v., respectively 3 h and 4 h after aspirin (10 mg kg^{-1}) or the vehicle used to dissolve aspirin. Animals were killed at 6 h. EGF did not significantly affect PGI_2 synthesis by tissues from animals that were not treated with aspirin (Table 2). However, it significantly increased the recovery of PGI_2 synthesis by all tissues, other than pericardium and ileum, from animals treated with aspirin (Figure 2). The effect was most marked on endothelial PGI_2 synthesis, which was 72 ± 11% control compared to 24 ± 7% control in

Table 2 Tissue 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) synthesis and serum thromboxane B_2 (TXB_2) concentration in the rabbit

Tissue 6-oxo-$PGF_{1\alpha}$ production	*Vehicle alone[a]*	*Vehicle plus cycloheximide[b]*	*Vehicle plus EGF[c]*
Aortic endothelium (pg cm^{-2} min^{-1})	97 ± 15	378 ± 69*	85 ± 11
Aortic media/adventitia (pg mg^{-1} min^{-1})	8.9 ± 1.9	10.3 ± 1.7*	6.5 ± 0.4
Pericardium (pg mg^{-1} min^{-1})	1.9 ± 0.4	8.4 ± 1.8*	2.2 ± 0.7
Peritoneum (pg mg^{-1} min^{-1})	1.5 ± 0.6	3.7 ± 1.1*	2.4 ± 0.6
Ileum (pg mg^{-1} min^{-1})	2.2 ± 0.9	27.7 ± 13.6*	3.1 ± 0.6
Gastric mucosa (pg mg^{-1} min^{-1})	6.3 ± 2.0	12.9 ± 2.9*	4.3 ± 0.7
Cardiac ventricle (pg mg^{-1} min^{-1})	1.1 ± 0.3	1.9 ± 0.2*	1.4 ± 0.1
Serum TXB_2[d] (ng ml^{-1})	202.4 ± 19.7	—	205.1 ± 22.1

[a]Animals killed 6 h after vehicle ($n = 6$).
[b]Animals killed 6 h after vehicle, injected with cycloheximide as described in the text ($n = 6$). Serum TXB_2 was not measured.
[c]Animals killed 6 h after vehicle, injected with epidermal growth factor (EGF) as described in the text ($n = 6$).
[d]Serum prepared by allowing blood to clot at 37°C as described in the text.
*Significantly different from vehicle alone, $P < 0.05$.

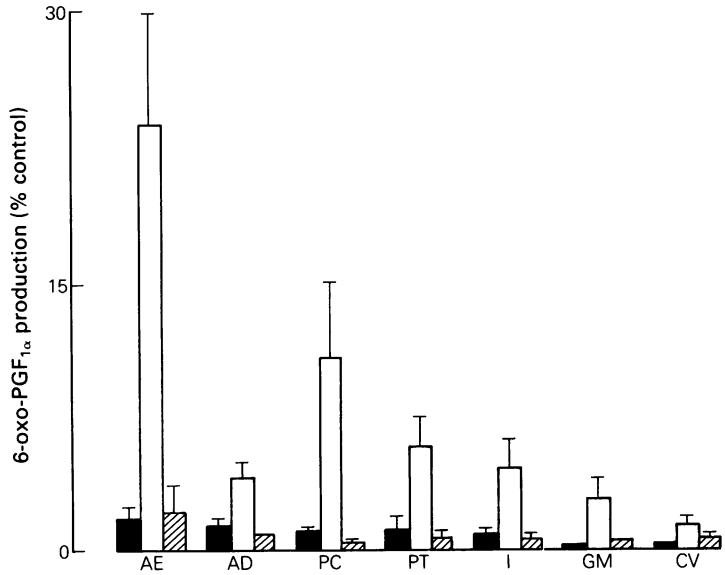

Figure 1 Effect of cycloheximide on recovery of tissue 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) production after aspirin. Columns (with s.e. mean) indicate mean 6-oxo-$PGF_{1\alpha}$ production after aspirin (10 mg kg^{-1}, i.v.). Results 0.5 h after aspirin are shown by the solid columns, 6 h after aspirin by the open columns. The hatched columns show 6-oxo-$PGF_{1\alpha}$ production 6 h after aspirin in animals treated with cycloheximide (200 mg kg^{-1}). AE: aortic endothelium; AD: aorta denuded of endothelium; PC: pericardium; PT: peritoneum; I: ileum; GM: gastric mucosa; CV: cardiac ventricle. The partial recovery at 6 h was abolished by cycloheximide.

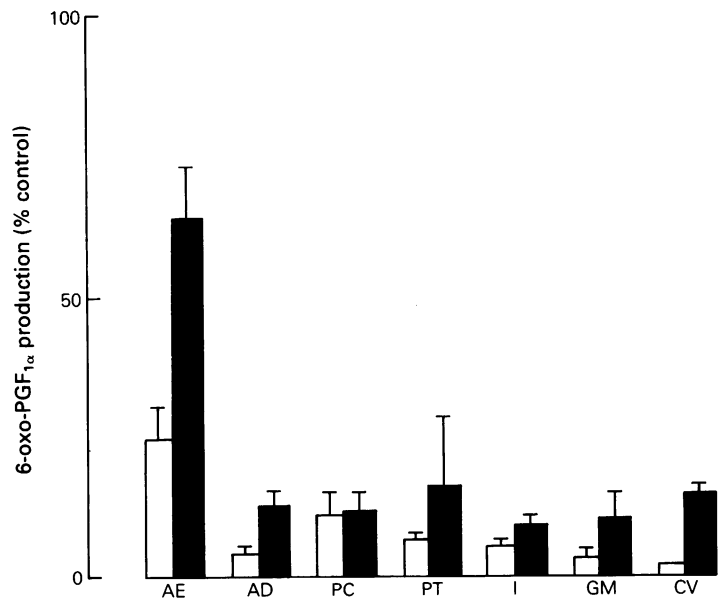

Figure 2 Effect of epidermal growth factor (EGF) on recovery of tissue 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) production after aspirin. Columns indicate mean 6-oxo-$PGF_{1\alpha}$ (with s.e. mean) 6 h after aspirin (10 mg kg^{-1} i.v.). The open columns show values in control animals treated with aspirin alone, the solid columns show results after aspirin and EGF (3 $\mu g\,kg^{-1}$). AE: aortic endothelium; AD: aorta denuded of endothelium; PC: Pericardium; PT: peritoneum; I: ileum; GM: gastric mucosa; CV: cardiac ventricle. 6-oxo-$PGF_{1\alpha}$ production was significantly increased by EGF in each tissue except pericardium and ileum.

animals treated only with aspirin. The concentration of TXB_2 in serum was not significantly affected by prior treatment of the animals with EGF.

Discussion

Thirty minutes after treatment of rabbits with aspirin, PGI_2 synthesis is inhibited profoundly and to a similar extent in all tissues (Table 1). The more rapid subsequent recovery of PGI_2 production by aortic endothelium than by other tissues, including aorta denuded of endothelium, accords with our hypothesis that cyclo-oxygenase is synthesized more rapidly in endothelium than in deeper vascular layers (Heavey *et al.*, 1985). However, the rate of recovery of aortic endothelial PGI_2 synthesis observed in the present experiments is not as great as the rate of recovery of bradykinin-stimulated PGI_2 synthesis observed in man by Heavey *et al.* (1985). This difference is not due to slow elimination of aspirin in the rabbit (Buchanan *et al.*, 1983). In interpreting the present experiments, it should be noted that while the template method maximizes the endothelial contribution of 6-oxo-$PGF_{1\alpha}$ in the sample, some PGI_2 will diffuse from deeper layers into the incubation medium. This unavoidable methodological limitation will tend to reduce the difference observed between aortic endothelium and aorta denuded of endothelium, causing the rate of recovery of endothelial PGI_2 synthesis to be underestimated. It must also be noted that each tissue contains endothelium, by virtue of its blood supply, further complicating the analysis.

An alternative explanation for the more rapid recovery of endothelial PGI_2 synthesis from inhibition by aspirin than that of other tissues, is that this reflects some pharmacokinetic property of aspirin. For instance, if aspirin were to persist longer within the media of the aorta than in the blood stream, then inhibition would last longer in chopped aorta than in endothelium. It is difficult to test this possibility directly because of the ubiquity of highly active tissue and plasma esterases that hydrolyse aspirin (see Williams, 1985), and because salicylate (to which aspirin is hydrolysed) is itself a reversible inhibitor of cyclo-oxygenase (Vane 1971). However, the effects of cycloheximide and EGF described here argue against such an explanation, because there is no reason to expect that these compounds would alter acutely the metabolism of aspirin, yet they do affect the recovery of PGI_2 synthesis after inhibition by aspirin. Cycloheximide abolishes the partial recovery of PGI_2 synthesis 6 h after aspirin (Figure 1), suggesting that recovery depends on synthesis of new enzyme. Conversely, EGF, which increases the rate of synthesis of cyclo-oxygenase in aspirin-treated cells in culture (Bailey *et al.*, 1985), also increased recovery of PGI_2 synthesis in the present experiments (Figure 2), in which it was administered *in vivo*.

Thus, in the rabbit *in vivo*, recovery of the capacity to synthesize PGI_2 following intravenous aspirin, occurs more rapidly in aortic endothelium than in deeper vascular layers, depends upon new protein synthesis and is accelerated by EGF. The simplest explanation is that cyclo-oxygenase is synthesized more rapidly in endothelium than in other tissues, in particular vascular media and adventitia. This conclusion accords with observations by Weksler *et al.* (1985), who found that endothelial PGI_2 synthesis by human saphenous vein recovers more rapidly from inhibition by aspirin than does PGI_2 synthesis by chopped venous fragments. Such differences in the rates of enzyme synthesis could also explain the observation of de Witt *et al.* (1983), that while PGI_2 synthase is uniformly distributed through the wall of bovine aorta, cyclo-oxygenase is some 20 times more concentrated in endothelium than in media.

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