Inhibition of prostaglandin synthesis does not affect contractile responses to noradrenaline, serotonin, angiotensin II nor endothelin-1 in human forearm isolated veins

JAYE P. F. CHIN, ROBYN M. HURLSTON & ANTHONY M. DART

Alfred and Baker Medical Unit, Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

- 1 The functional role of endogenous cyclo-oxygenase products was examined in the human forearm isolated vein.
- 2 Six healthy normal adult males (age > 18 years old) were recruited. Forearm veins were biopsied and ring segments mounted in standard organ baths for recording of isometric force.
- 3 Noradrenaline ($-\log$ molar EC_{50} : 7.75 ± 0.19 ; $-\log$ molar threshold concentration: 8.80 ± 0.20), 5-hydroxytryptamine ($-\log$ molar EC_{50} : 7.52 ± 0.17 ; $-\log$ molar threshold concentration: 9.50 ± 0.64), angiotensin II ($-\log$ molar threshold concentration: 9.00 ± 0.28) and endothelin-1 ($-\log$ molar threshold concentration: 9.13 ± 0.47) were equipotent in this preparation. Indomethacin ($10 \mu\text{M}$) had no effect on either the threshold concentration or EC_{50} of noradrenaline, 5-hydroxytryptamine nor the threshold concentration of angiotensin II nor endothelin-1.
- 4 Sodium nitroprusside (1 nM – $10 \mu\text{M}$) relaxed noradrenaline-precontracted preparations. Evidence of minimal endothelial influence was confirmed by the lack of relaxant response to acetylcholine (1 nM – $10 \mu\text{M}$). Histology using silver staining confirmed that endothelial cells were absent over greater than 90% of the lumen surface.
- 5 We conclude that endogenous prostanoids derived from smooth muscle cells, either released basally or agonist-stimulated, do not play a role in the regulation of vascular tone in the human forearm isolated vein.

Keywords cyclo-oxygenase products prostacyclin human forearm vein smooth muscle

Introduction

Prostacyclin (prostaglandin I_2 ; PGI_2), the major prostanoid formed in smooth muscle [1], is derived from the prostaglandin endoperoxide PGH_2 , a direct product of cyclo-oxygenase activity. Although early studies suggested differential rates of prostacyclin formation along the layers of the vasculature (endothelial cells having considerably greater synthase activity than smooth muscle cells [2]) it has since been shown that smooth muscle cells cultured from rabbit [3], pig [4] and human [5] are capable of synthesizing prostacyclin at rates comparable with endothelial cells. The question arises as to the functional role of smooth muscle derived cyclo-oxygenase products.

Endogenous release of a dilatatory prostanoid from

smooth muscle cells has been shown to modulate contractile responses of rat aorta [6], human femoral artery strips [7] and human saphenous veins [8]. On the other hand, cyclo-oxygenase products are reported not to be involved in the endothelin-1 response in human hand vein [9]. However, since the ratio of PGI_2 to PGE_2 production by freshly isolated porcine smooth muscle cells decreases from a value of 5 after 5 h of culture to 0.9 by 48 h [4], and reactivity of veins was examined up to 24 h after harvesting in the study of Arner & Hogestatt [9], it is conceivable that the non-effect of indomethacin was due to the decreased production of PGI_2 at the time of study. In the present paper we examined the interaction between endogenous cyclo-

oxygenase products and responses to contractile stimuli or forearm venous smooth muscle studied within the first hour of harvesting.

Methods

Six healthy males (age >18 years old) were recruited for participation. Criteria for inclusion was non-smoking status, absence of alcohol abuse (<2 standard alcohol drinks/day), systolic blood pressure <140 and diastolic blood pressure <90 mm Hg, total serum cholesterol <5.5 mmol l⁻¹ and total triglyceride <2.0 mmol l⁻¹. In addition all volunteers had normal findings on physical examination as well as routine haematology and biochemical blood analyses. Subjects were instructed to refrain from NSAIDs medication during the month before each biopsy took place. This study was approved by the Alfred Group of Hospitals Ethics Committee and written informed consent was obtained from all subjects.

The methods used are as described elsewhere [10]. In brief, a 1 cm *in vivo* length of human forearm vein was biopsied and immediately cut into 2 or 3 ring segments. Each ring was quickly set up for recording of isometric wall force (g) in a 25 ml siliconized, water-jacketed organ bath containing Krebs' solution (mm: Na⁺ 144, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, H₂PO₄²⁻ 1.2, Cl⁻ 128.7, HCO₃²⁻ 25, SO₄²⁻ 1.2, glucose 11, ethylenediamine tetra-acetic acid (EDTA) 0.027) bubbled with carbogen (95% O₂, 5% CO₂) and maintained at 37° C. After an equilibration period of 30 min, each ring was subjected to a length-tension curve to normalise the internal circumference at the level of passive stretch equivalent to 0.9 × the transmural pressure of 20 mm Hg [10]. This was important to ensure that veins of different internal diameter were stretched passively to a similar point on their length-tension curve.

Figure 1 Flow-chart of experimental protocols 1 and 2. Each agonist was washed out thoroughly with Krebs' solution and a 30 min resting period was observed between each concentration-response curve. The effect of indomethacin on 5-HT and noradrenaline was analysed within protocol 1, while protocol 2 acted as time-controls. The effect of indomethacin on angiotensin II and endothelin-1 was analysed across protocols.

Protocol 1	Protocol 2
normalisation procedure	normalisation procedure
high K ⁺ solution	high K ⁺ solution
noradrenaline CR curve	noradrenaline CR curve
5-HT CR curve	5-HT CR curve
indomethacin (10 µM)	
angiotensin II CR curve	angiotensin II CR curve
indomethacin (10 µM)	
noradrenaline CR curve	noradrenaline CR curve
indomethacin (10 µM)	
5-HT CR curve	5-HT CR curve
indomethacin (10 µM)	
endothelin-1 CR curve	endothelin-1 CR curve

After a further 30 min, rings from each subject underwent one of two protocols. Figure 1 depicts a flow-chart of the protocols used. All vessels were initially contracted with high potassium containing physiological saline solution (KPSS; K⁺ 124 mM). In protocol 1, cumulative concentration-response curves in log concentration progression ratios of 0.5 were constructed to noradrenaline (NA; 1 nM–10 mM) and 5-hydroxytryptamine (5-HT; 1 nM–10 mM). After a 30 min incubation with indomethacin (10 µM) these curves were repeated in the presence of indomethacin. Since angiotensin II (AII) displays tachyphylaxis at high concentrations [10], only a single curve per ring was obtained for this agonist. In protocol 1, this was done in the presence of indomethacin. Similarly, due to drug limitation, only a single concentration-response curve was constructed to endothelin-1 (ET-1) and, in protocol 1, this was done in the presence of indomethacin. Protocol 2 served as the time control experiment. Hence all concentration-response curves were constructed in protocol 2 as in protocol 1 with the exception that indomethacin was not added in this series of experiments. Where a third vessel was obtained, endothelial cell function was examined. After obtaining a constriction to KPSS, a cumulative concentration-response curve to NA was obtained. From this a submaximal concentration was chosen and used to pre-constrict the vessel. Cumulative concentration-response curves to sodium nitroprusside (SNP; 1 nM–10 µM) and acetylcholine (ACh; 1 nM–10 µM) were then obtained. It should be noted that in all the protocols used, a 30 min re-equilibration period was observed between each concentration-response curve.

Silver staining was done in at least one ring from each segment. This was performed at the end of the organ bath procedure. After preparing the rings in a 5% v/v glutaraldehyde solution and thorough rinsing in 5% w/v glucose solution, the tissues were exposed to 15 mM silver nitrate and mounted onto a standard microscope slide ready for stain development with either fluorescent or u.v. light illumination. Segments were then viewed under a Nikon TMD Diphot microscope and images were photographed onto black and white film with an attached 35 mm camera.

Statistical analysis

Data are presented as mean ± s.e. mean and analyzed using Student's *t*-test (paired where appropriate). *P* < 0.05 is used as the criterion for statistical significance.

Contractile responses were measured as grams force normalised to the internal diameter. Since a maximum response was not achieved either to AII or ET-1, responses to both agonists are expressed as a percentage of the KPSS response. Negative log (–) molar EC₅₀ concentrations (concentration of drug giving a 50% response) were obtained from individual concentration-response curves to NA, 5-HT and SNP that were fitted to a logistic equation of the form $E = MA^P / (A^P + K^P)$ where *E* is the response, *M* is the maximum response, *A* is the concentration eliciting *E*, *K* is the concentration eliciting 50% of the maximum response and *P* is the slope parameter [11].

Drugs used

Drugs used were (–) noradrenaline bitartrate (Sigma), 5-hydroxytryptamine creatinine sulphate complex (serotonin, Sigma), angiotensin II (hypertensine, Ciba-Geigy), indomethacin (Sigma), acetylcholine bromide (Sigma) and sodium nitroprusside (David Bull Laboratories Pty Ltd). Stock solutions of all drugs (with the exception of indomethacin) were made up on the day of study and diluted to the appropriate concentration in milli-Q distilled water. Indomethacin was dissolved in 0.1 M Na₂CO₃.

Results

From the six vein segments biopsied (mean length *in vivo* 10.97 ± 3.04 mm; mean length after recoil 4.71 ± 1.31 mm), 14 viable rings were obtained. At least 2 rings were obtained from all but one segment (where only one ring was viable) and from three segments, three rings were procured. Mean *in vitro* ring length was 1.85 ± 0.51 mm and mean ring diameter at L₂₀ was 2.95 ± 0.89 mm.

KPSS elicited contractile responses of 1.51 ± 0.62 g mm⁻¹ (diameter) in rings undergoing protocol 1 and 1.25 ± 0.63 g mm⁻¹ in rings undergoing protocol 2.

NA, as did 5-HT, dose-dependently contracted this preparation from concentrations of 1 nM to 0.1 mM. The concentration-response curve to AII was bell-shaped supporting preliminary observations that AII was tachyphylactic at high doses in this preparation. Comparison of threshold concentrations (minimum concentration at which a response could be elicited) demonstrated that all the agonists used were equipotent in this preparation (Table 1).

Indomethacin had no effect on either the sensitivity or range to NA (Figure 2), 5-HT, AII nor ET-1 (Table 1). Any slight shift observed in the concentration-response curves to NA and 5-HT in the presence of

Figure 2 Indomethacin (10 mM) had no effect on the mean concentration-response curve to noradrenaline alone (○) and with indomethacin (●). Vertical lines depict s.e. mean.

indomethacin could be accounted for by the time-control experiments (Table 1).

While SNP induced concentration-dependent dilatation in each of the three 'extra' rings procured (Figure 3a), the endothelium-dependent agonist ACh had little or no relaxant effect. In two of the three rings some contraction was observed (Figure 3b). Silver staining revealed that less than 10% of the endothelial cells were still intact in these rings (Figure 4).

Discussion

We conclude that PGI₂ from smooth muscle does not contribute to the regulation of vascular tone in the human forearm vein. Indeed, the current study provides no functional evidence that prostacyclin synthesis takes place in the smooth muscle of this preparation. This conclusion, however, requires confirmation from biochemical measurements of cyclo-oxygenase products. Our main conclusion stems from

Table 1 Effect of indomethacin (10 µM) (and time) on negative log EC₅₀, negative log threshold concentrations and maximum responses to the agonists used

Agonists	Negative log EC ₅₀	Negative log threshold concentration	Maximum force (g mm ⁻¹)
Noradrenaline (P1)	7.75 ± 0.19	8.80 ± 0.20	1.92 ± 0.11
+ indomethacin (P1)	7.31 ± 0.15	9.00 ± 0.60	1.90 ± 0.13
Noradrenaline (P2)	7.77 ± 0.19	8.50 ± 0.35	1.56 ± 0.39
+ 30 min (P2)	7.05 ± 0.09	8.50 ± 0.35	1.58 ± 0.39
5-HT (P1)	7.52 ± 0.17	9.50 ± 0.64	2.34 ± 0.30
+ indomethacin (P1)	7.32 ± 0.15	8.90 ± 0.71	2.19 ± 0.22
5-HT (P2)	7.35 ± 0.09	8.50 ± 0.20	1.87 ± 0.43
+ 30 min (P2)	7.24 ± 0.12	8.00 ± 0.35	1.80 ± 0.40
Angiotensin II (P2)	—	9.00 ± 0.28	—
+ indomethacin (P1)	—	8.75 ± 0.14	—
Endothelin-1 (P2)	—	9.13 ± 0.47	—
+ indomethacin (P1)	—	9.88 ± 0.55	—

P1—from protocol 1.

P2—from protocol 2.

Figure 3 Sodium nitroprusside (SNP; 1 nM–30 μM; 3a) caused full relaxation on vessels precontracted with noradrenaline (NA). Acetylcholine (ACh) had little or no relaxant effect and induced constriction at high concentrations (>1 μM; 3b). W is washout.

Figure 4 The presence of endothelial cells on the isolated forearm vein preparation was determined by silver staining. In a typical preparation at least 90% of the lumen was denuded of these cells. This image corresponds to a section (approximately 25%) of a forearm vein ring that had undergone silver staining, depicting that <10% of endothelial cells still intact. This is the only section in which endothelial cells were observed. Magnification ×20.

the finding that the cyclo-oxygenase inhibitor indomethacin had no effect on the contractile responses to any of the agonists used demonstrating that neither basal nor stimulated release of PGI₂ (or any other cyclo-oxygenase products) from venous smooth muscle

influences vascular reactivity. Time-control experiments confirmed the reproducibility of the responses to the agonists. The alternate interpretation of our data is that the effect of PGI₂ released from vascular smooth muscle cells is balanced by release of cyclo-oxygenase dependent constrictor prostaglandins. Without experiments using specific receptor antagonists, for example to thromboxane A₂, this cannot be answered.

Although deliberate de-endothelialising was not performed on these vessels, it soon became evident from both the functional experiments with acetylcholine (performed in three rings) and the histology performed using silver staining (performed on at least one ring from each segment) that a large amount of the endothelial layer was lost during harvesting and/or mounting of the vein, supporting the findings of Sayers *et al.* [12] that the inevitable spasm of veins during procurement often leads to endothelial cell loss. Since rings were able to dilate with sodium nitroprusside, it is clear that the lack of relaxant response to acetylcholine was not due to the inability of the vessel to relax, rather that endothelial cell damage had occurred limiting the acetylcholine-induced release of nitric oxide [13]. Thus from our series of experiments it is not possible to relate the extent to which synthesis and/or release of prostacyclin from the endothelium contributes to the regulation of vascular tone. From superficial forearm veins of patients undergoing hand surgery where the endothelium was still intact [9] denudation of this layer did not alter contractile responses to noradrenaline, serotonin or PGF_{2α} suggesting perhaps that prostanoid synthesis from the endothelial cells of this preparation is functionally unimportant.

It has been demonstrated that there is diminished prostacyclin formation in rat veins compared with arteries [14]. This may not be true of human vascular smooth muscle preparations, however, since both arterial and venous preparations have been shown to be indomethacin-modulated. In helically cut human femoral artery strips denuded of endothelium, indomethacin potentiated the contractile responses to PGF_{2α} by 60% suggesting some synthesis, and influence, of a relaxant prostanoid in this smooth muscle preparation [7]. Similarly, in spirals of human saphenous veins, indomethacin was demonstrated to potentiate the contractile activity of endothelin-1 [8], suggesting that the lack of effect of indomethacin in the present study may be particular to the forearm vein. This is further supported by the findings of Haynes & Webb [15] who were able to demonstrate a potentiation of endothelin-1 induced venoconstriction by aspirin *in vivo* in the human dorsal hand vein. It is unlikely that the agonists used in the current study are responsible for any lack of prostanoid stimulatory action in the hand vein since AII-induced prostanoid synthesis in vascular smooth muscle preparations is well-documented [16, 17] as is the stimulation by 5-HT [18] and endothelin-1 [8]. It has also been demonstrated that prostaglandin synthesis elicited by adrenergic stimuli in cultured smooth muscle cells of rabbit aorta is potently promoted by noradrenaline [19].

It is apparent from our data that the contribution of prostanoids, either basally-released or agonist-

stimulated, from human forearm vein on the regulation of vascular tone is minimal. The present paper supports the finding of Arner & Hogestatt [9] that the contraction by endothelin-1, and indeed all the other agonists used is not modulated by an endogenous prostanoid release.

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