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Papaverine Effects on PGI_2 and TXA_2 Release from the Canine Vascular Wall

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ABSTRACT. **Operative manipulation of blood vessels might lead to spasm, thereby destroying the endothelial cell function: the spasm can be prevented by the vasodilator papaverine. To study if this was mediated via the prostanoid pathway the following investigation was undertaken: canine jugular veins and carotid arteries were dissected with or without papaverine. Vessel segments were then perfused with Hank's balanced salt solution for five times 15 min. Prostacyclin was measured as the stable degradation product 6-keto-$PGF_{1\alpha}$ and thromboxane as TXB_2, by radioimmunoassay. Control arterial segments' 6-keto-$PGF_{1\alpha}$ release was initially 129.5 + 20.1 pg/mm²/15 min, and 29.7 + 10.4 after 60 min ($p<0.05$ vs initial value) and responded to arachidonic acid (AA) with an increase to 139.2 ± 23.1 pg/mm²/15 min ($p<0.05$). Segments treated with papaverine had the same release as the controls. In venous segments there was a lower initial release ($p<0.05$) from segments given papaverine than from controls, but this was more likely an effect of papaverine on the assay. There was no difference in release of prostacyclin from segments given papaverine in the perfusate compared to controls when using ^{125}I tracer. When using ^{3}H tracer including absorption of free antigen to dextran coated charcoal, papaverine displaced the free tracer giving artificially low values. There was no effect of papaverine given intraoperatively on the TXB_2 release, neither from arteries nor from veins. In another experiment the vessel wall tension was examined and the cyclooxygenase inhibitor diclofenac did not inhibit the vasodilating effect of papaverine. It is concluded that the vasodilating effect of papaverine is not mediated via prostacyclin release and that the vasodilating effect does not influence the prostacyclin release from arteries or veins. Finally, papaverine interacts with the ^{3}H assay by displacing the antigen-antibody complex.**

INTRODUCTION

Vein grafts are sometimes complicated by thrombosis although not as often as synthetic grafts used in the same position. One reason suggested for the development of thrombosis has been that spasm of the harvested vein disturbs the endothelial cell function with interruption of the endothelial cellular line as well as destruction of the endothelial cells (1–3). Harvested veins have displayed endothelial cellular disruption and intimal thickening (4–6). Endothelial denudation increases intimal thickening (7). Papaverine acts as a smooth muscle relaxant and the use of papaverine before vein harvest decreased endothelial damage and intimal thickening (8–10). Theoretically this relaxation could be mediated through the arachidonic acid pathway. The two prostanoids prostacyclin and thromboxane are both involved in the regulation of thrombosis and haemostasis. Prostacyclin is the most potent vasodilator and inhibitor of platelet aggregation known and is the main prostanoid from the endothelial cell (11). Thromboxane is a potent vasoconstrictor and platelet aggregator and is mainly derived from platelets (11) but also from the vascular wall (12). Papaverine has been reported to block the release of phospholipids in platelets (27).

Perfusion of vessels ex vivo gives rise to an initial burst in prostacyclin and thromboxane release with a rapid decline and relatively stable release for 3 h (13–14). If AA (arachidonic acid) is provided to the perfusate, either immediately, after 1 h or after 6 h, there is an increased release of prostanoids (14).

The objective of this investigation was to study if papaverine, given perioperatively or directly to the perfusate, would influence the prostanoid release from the vascular wall in ex vivo perfused canine arteries and veins, and if any inhibitory action was at the

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phospholipase level. Furthermore, the aim was to study if the dilating effect of papaverine could be blocked by a cyclooxygenase inhibitor.

MATERIALS AND METHODS

Operative procedure

Six adult mongrel dogs were anesthetized with pentobarbital (20 mg/kg bw), intubated and mechanically ventilated. External jugular veins and carotid arteries were exposed through a midline neck incision. On one side 5–10 ml papaverine hydrochloride (0.6 mg/ml; Sigma) was injected around and into the vascular sheath, without penetrating the vessel. The vessel was gently dissected free and all tributaries were ligated. On the contralateral side, exactly the same procedure was performed but without administration of papaverine. Seven other mongrel dogs were used for the study of the effect of papaverine given directly to the perfusate and were operated upon as when harvesting the control segments. Animal care in this study was carried out in accordance with the 'Principles of Laboratory Animal Care' and 'Guide for the Care of and Use of Laboratory Animals' (NIH Publication No 80–23, revised 1978). The vessels were then rinsed and flushed with room temperate calcium and magnesium-free Hank's balanced salt solution (CMF-HBSS; Gibco Laboratories, Grand Islands, NY, pH 7.4, 290 mOsm) until the effluent was clear. After excision, veins retracted to about half of their in situ length, whereas the arteries retracted to two-thirds with no difference when papaverine was given. Each vessel was cut into 3 cm length, and briefly stored in CMF-HBSS until subsequently being perfused. The circumference and length of the retracted segments were measured in order to calculate the perfused area, allowing prostanoid release to be adjusted for luminal surface area.

Perfusion technique

The open recirculating perfusion device has previously been described in detail (15, 16). Briefly, a pulsatile pump created a pulsatile flow that, when creating venous flow, was converted by a 'windkessel device' to a nonpulsatile flow. With a resistance clamp inserted after the vessel segment, a desired pressure was built up in the system and measured by a pressure recorder. The vessels were mounted in the perfusion system and immersed in a 37 °C HBSS bath. The segments were expanded to approximately their in situ length, but not overstretched. Control segments and experimental segments were perfused simultaneously, arteries and veins being randomly perfused as pair number one and two. Veins were perfused at a pressure of 7 mmHg with a mean nonpulsatile flow of 90 ml/min, whereas arteries were perfused at a pressure of 100 mmHg with a mean pulsatile flow of 90 ml/min.

All segments were perfused with HBSS (37 °C, pH 7.4, 290 mOsm) for 5 × 15 min with the exchange of perfusate after each time period. To the last perfusate, AA was given reaching a final concentration of 4 μg/ml. The perfusates were stored at –70 °C until subsequent radioimmunoassay (RIA). To study the effect of papaverine given to the perfusate, this agent was given in a dose of 15 mg providing a concentration of 0.6 mg/ml.

Extraction procedure

Samples were extracted prior to radioimmunoassay to remove lipids and other potential interferents using a modification of procedures previously described (17). An aliquot of ^{3}H-TXB_2 (100 μl, 0.02nCi; NET-603, New England Nuclear, Boston, MA) was added to each sample (1.0 ml) to monitor the extraction efficiency. An aliquot of acetonitrile (1.0 ml) was then added to each sample and the protein precipitate which forms was discarded. The supernatant was acidified with formic acid (0.1 ml, pH 3.5) and washed with petroleum ether (2 × 3 ml). The eicosanoids were then extracted from the aqueous layer with ethyl acetate (2 × 4 ml) The extract was air-dried, stored at –20 °C and redissolved in RIA assay buffer (1.0 ml; 0.1% pigskin gel, 1M PO_4, 0.15M NaCl, pH 7.4) just before analysis. The efficiency of extraction of ^{3}H TXB_2 calculated from the beta radioactivity levels in a 0.1 ml aliquot of extracted sample, was used to correct the results of the subsequent prostaglandin immunoassays.

3H Radioimmunoassay

6-Keto-$PGF_{1\alpha}$, the stable degradation product of prostacyclin, was measured using a commercially available reagent kit (NEK-008, New England Nuclear, Boston, MA). The limit of detection in the analysis was 59 pg/ml (±2 SD from the response in the absence of standard). According to the manufacturer, the relative potency of other prostaglandins, calculated from the concentrations required to reduce the binding of the tracer by 50% was in percentages: PGE_2 2.5, $PGF_{1\alpha}$ 0.3, PGE_1 0.2, $PGF_{2\alpha}$ 0.05 and TXB_2 <0.3 (manual New England Nuclear).

TXB_2 was determined using a similar method employing antibodies (a generous gift of the Upjohn Company, Kalamazoo, MI) produced according to published methods (19) and a commercially available tritiated TXB_2 tracer (NET-603, New England Nuclear). The detection limit with this method was 11.8 pg/ml. The relative binding potency of other prostaglandins was in percentages: PGD_2 1.0, PGD_1 1.0, $PGF_{1\alpha}$ 0.04 and PGF_2 0.04.

^{125}I Radioimmunoassay

6-Keto-$PGF_{1\alpha}$ was also measured using a second commercial kit (NEK-025, New England Nuclear) which employs a ^{125}I-labelled derivative of 6-keto-$PGF_{1\alpha}$ instead of the tritiated tracer in the method previously

described. The sensitivity of this assay was 8.7 pg/ml and according to the manufacturer the cross-reactivity of other prostaglandins was in percentages: $PGF_{2\alpha}$ 2.6, PGE_1 1.9, TXB_2 1.4, PGE_2 1.1, $PGF_{1\alpha}$ 0.8, PGA_1 0.2, PGD_2 0.2, PGA_2 0.04, AA 0.005.

High pressure liquid chromatography

Reversed phase high pressure liquid chromatography (HPLC) according to a modification of methods previously described (20, 21) was used to confirm the identity of immunoactivity with pure standard 6-keto-$PGF_{1\alpha}$ and to further purify some samples. Samples (1 ml) were extracted according to the method described above and redissolved in HPLC elution solvent (200 µl) described below. The entire sample was subsequently applied to a 4 µm C-18 column (3.9 × 150 mm Novopak, Waters, Milford, MA) and isocratically eluted at ambient temperature with acetonitrile/water (31:69 v/v with 1% TFA) at a flow rate of 75 ml/min. The absorbance at 195 nm was continually monitored (SpectraFlow 773, Kratos Analytical Instruments, Westwood, NJ) and five fractions collected every min, i.e. 150 µl/fraction (Gilson Micro fractionator, Gilson Medical Electronics, Middleton, WI). Fractions were air-dried, reconstituted in assay buffer, and appropriately diluted for subsequent analysis by RIA.

Vascular tension

A 2–3 mm thick slice of the rabbit carotid artery was mounted on a L-shaped metal prong connected to a Grass polygraph. The vessel segment was immersed in 95% O_2 and 5% CO_2 balanced Kreb's solution. A tension of 4 mN was applied and the vessels were allowed to rest for 90 minutes. After precontraction with K^+ a segment of the vessel was given papaverine as a dilatatory stimulus and the other was given the cyclooxygenase inhibitor diclofenac (Voltaren^R) prior to papaverine. The relaxation of the vessel was expressed in percentage of the maximal K^+ contraction.

Scanning electron microscopy

After perfusion, the vein specimens were perfusion fixed at 7 mmHg and the arterial specimens at 100 mmHg in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h followed by 24 h storage in cacodylate buffer. The specimens were then incubated in ethanol in increasing concentration up to 99.99% and then incubated in hexamethyldisilazane for dehydration for 10 min, dried in air and sputter-coated with gold-palladium (22). Five random areas in each specimen were investigated and the percentage coverage by endothelial cells was estimated and scored. No effort was made to describe the morphological appearance of the endothelial cells.

Statistics

Prostanoid data in this article are expressed as mean ± standard error pg/mm²/15 min. Analyses were performed with test of variance and the Wilcoxon signed rank test for paired data. A p value <0.05 was considered significant.

RESULTS

In segments given papaverine prior to dissection, no circumferential vasoconstriction was noted. The circumferential vasoconstriction in control segments, however, was relieved when the segments were mounted in the perfusion system.

Papaverine given prior to harvest

Arteries (Fig. 1; ^{3}H RIA)

With the onset of perfusion arterial control segments had a burst of 6-keto-$PGF_{1\alpha}$ release which reached 129.5 ± 10.1 pg/mm²/15 min and declined to the lowest value after 60 min ($p<0.05$ vs initial value). When AA was given there was an increase to the initial level ($p<0.05$ vs

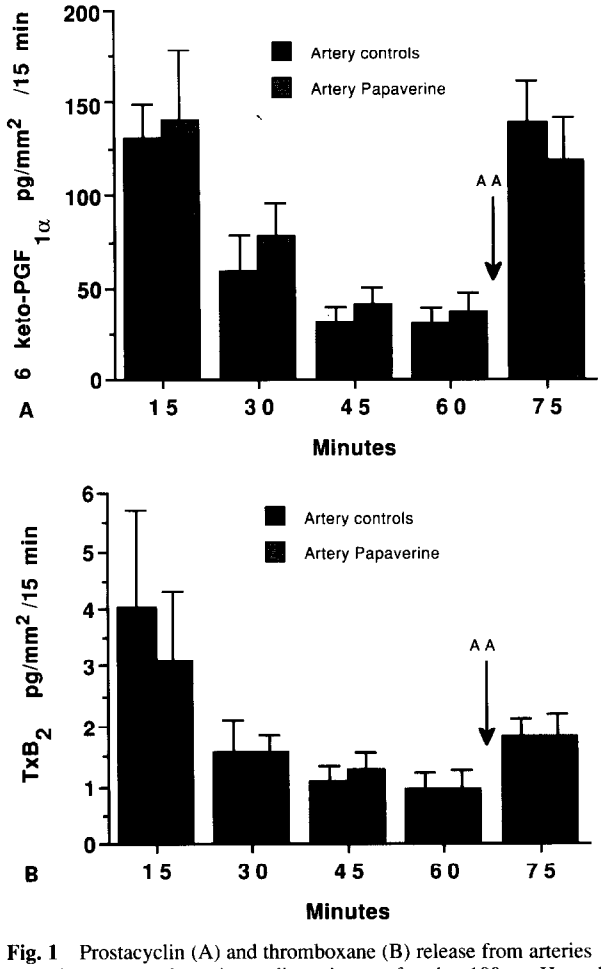


Fig. 1 Prostacyclin (A) and thromboxane (B) release from arteries (n6) given papaverine prior to dissection, perfused at 100 mmHg and with a mean pulsatile flow of 90 ml/min using ^{3}H RIA kit. AA, 4 µg/ml added. Mean ± SEM. No statistically significant difference between controls and papaverine at any point. Wilcoxon signed rank test for paired data.
*= $p<0.05$ vs initial value, **= $p<0.005$ vs 60 min value.

60 min value). From segments previously treated with papaverine the initial release was the same as from controls (ns vs control segments) declining to the lowest value after 60 min (ns vs control segments). When AA was given, there was a significant increase almost to the initial value (ns vs controls). TXB_2 release from controls was initially 4.0 ± 1.7 pg/mm²/15 min declining until 60 min (ns vs initial value). After AA administration the release was slightly increased but without reaching statistical significance. TXB_2 release from papaverine segments was not statistically significantly different from controls.

Veins (Fig. 2; ^3H RIA)
With the onset of perfusion the venous control segments' 6-keto-$PGF_{1\alpha}$ release was 84.3 ± 25.5 pg/mm²/15 min declining to the lowest value after 60 min ($p<0.01$ vs initial value). At that time when AA was given the release significantly increased to the initial value ($p<0.05$ vs 60 min value). The release from segments given papaverine was initially 44.3 ± 11.6 pg/mm²/15 min ($p<0.05$ vs controls) declining to the lowest value after 60 min (ns vs controls). When AA was given there was an increase to a level not significantly different from controls. TXB_2 release from control segments was 3.1 ± 1.2, 0.5 ± 0.1 and 1.2 ± 0.1 pg/mm²/15 min respectively, whereas the release from papaverine segments was 2.4 ± 0.6, 0.9 ± 0.3 and 1.2 ± 0.1 (not significantly different vs controls at any point).

Papaverine given in the perfusate

This study was only undertaken with *vein* segments. Using the ^3H RIA (Fig. 3) the initial 6-keto-$PGF_{1\alpha}$ release from control segments was 52.3 ± 13.4 pg/mm²/15 min declining until 60 min ($p<0.01$ vs initial value). It responded to AA with an increase to 95.4 ± 41.1 ($p<0.01$ vs 60 min value). Segments given papaverine into the perfusate had an initial 6-keto-$PGF_{1\alpha}$ release of 5.5 ± 2.5

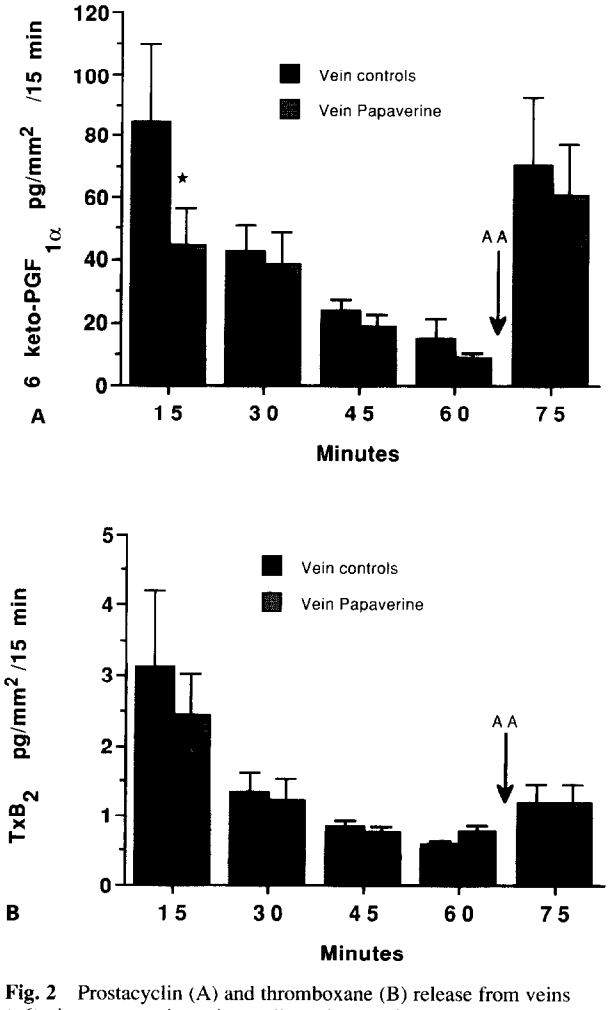


Fig. 2 Prostacyclin (A) and thromboxane (B) release from veins (n6) given papaverine prior to dissection, perfused at 7 mmHg and with a mean nonpulsatile flow of 90 ml/min. AA, 4 μg/ml added. Mean ± SEM.
*= $p<0.05$ (Wilcoxon signed rank test for paired data). **= $p<0.01$ vs initial value.

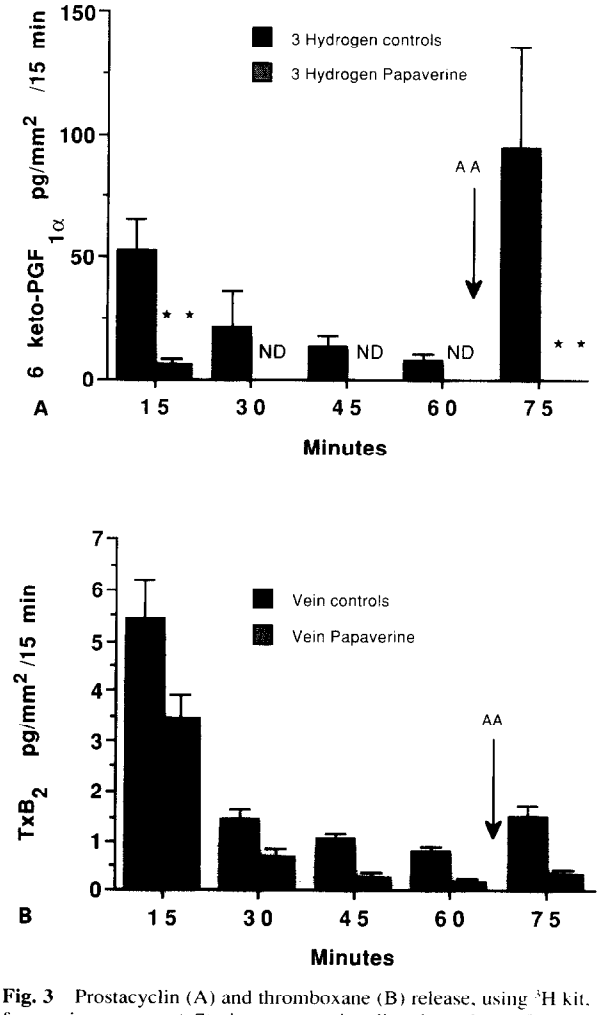


Fig. 3 Prostacyclin (A) and thromboxane (B) release, using ^3H kit, from vein segments (n7) given papaverine directly to the perfusate. AA, 4 μg/ml added. Mean ± SEM.
*= $p<0.05$, **= $p<0.01$ (Wilcoxon signed rank test for paired data). ND = not detectable.

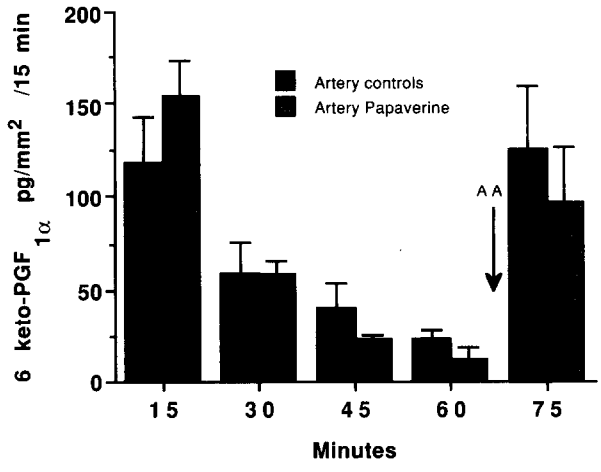


Fig. 4 Prostacyclin release, using ^{125}I kit, from vein segments (n7) given papaverine directly to the perfusate. AA, 4 μg/ml added. Mean ± SEM. No statistically significant difference at any point. (Wilcoxon signed rank test for paired data.)

($p<0.05$ vs controls) declining to undetectable amounts at 60 min. When AA was given there was no response with detectable amounts in only two experiments, (0.5 ± 0.3 pg/mm²/15 min) ($p<0.001$ vs controls). TXB_2 release from controls was initially 5.4 ± 0.8 pg/mm²/15, also declining until 60 min ($p<0.01$ vs initial value). It increased after addition of AA ($p<0.01$ vs 60 min value). From segments given papaverine the TXB_2 release was lower than from controls at all points ($p<0.05$).

When using the ^{125}I RIA (Fig. 4) the 6-keto-$PGF_{1\alpha}$ release from control segments was initially 117.8 ± 25.8 pg/mm²/15 min declining until 60 min ($p<0.05$ vs initial value). With AA the release increased to the initial value ($p<0.05$ vs 60 min value). The 6-keto-$PGF_{1\alpha}$ release from segments given papaverine to the perfusate elicited the same release as the control segments. No ^{125}I RIA for TXB_2 was undertaken. In a separate study,

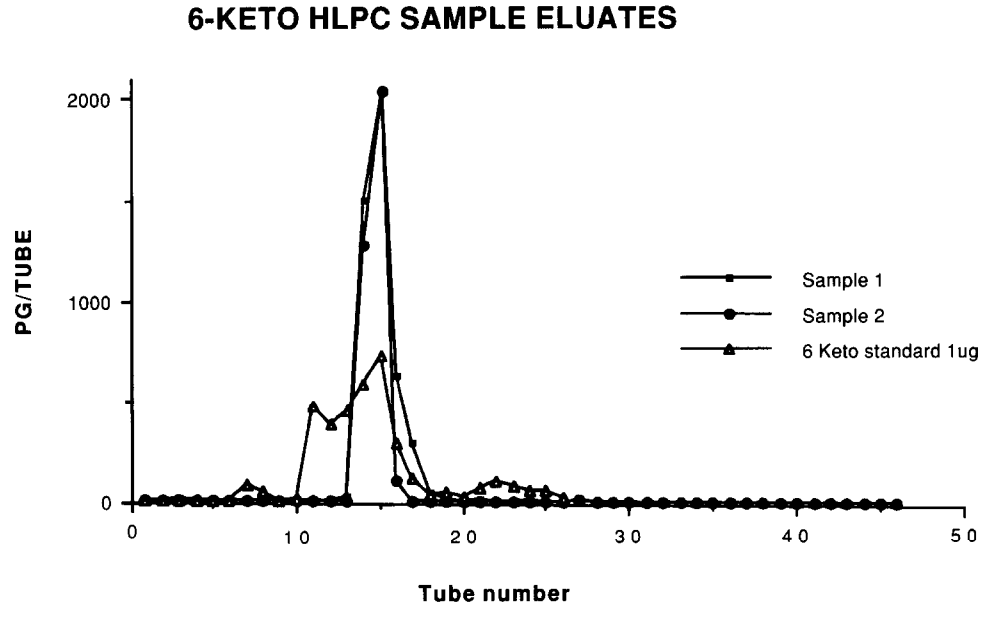


Fig. 5 HPLC of two test samples and known 6-keto-$PGF_{1\alpha}$ standard. No other prostanoids than the immunoactive 6-keto-$PGF_{1\alpha}$ peaks were detected in the test samples.

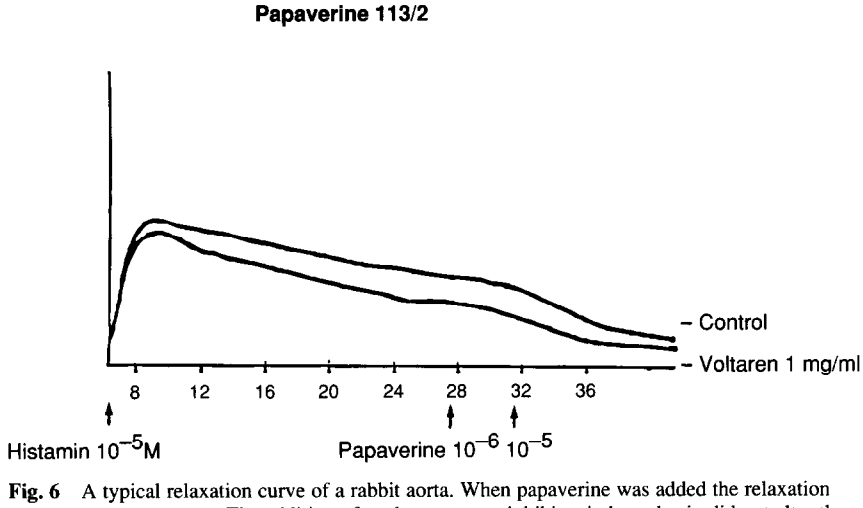


Fig. 6 A typical relaxation curve of a rabbit aorta. When papaverine was added the relaxation was marked (see arrow). The addition of cyclooxygenase inhibitor indomethacin did not alter the dilatatory effect of papaverine.

papaverine was added to the dextran charcoal solution which displaced the bound radioactivity into the liquid fraction to 100% of that in the absence of prostaglandin, so giving high radioactivity as if there was no detectable 6-keto-$PGF_{1\alpha}$.

High pressure liquid chromatography (Fig. 5)

The standard for the iodinated kit showed a somewhat broad spectrum with suspect double spike for the 6-keto-$PGF_{1\alpha}$, whereas the spike from the two samples corresponding to 6-keto-$PGF_{1\alpha}$ was much thinner. No other spikes were found.

Vascular tension

A typical effect of papaverine is noted in Figure 6. The vessels relaxed spontaneously, but this was accelerated to different extents in the various segments when papaverine was added. Segments pretreated with cyclooxygenase inhibitors did not alter the dilatatory effect of papaverine.

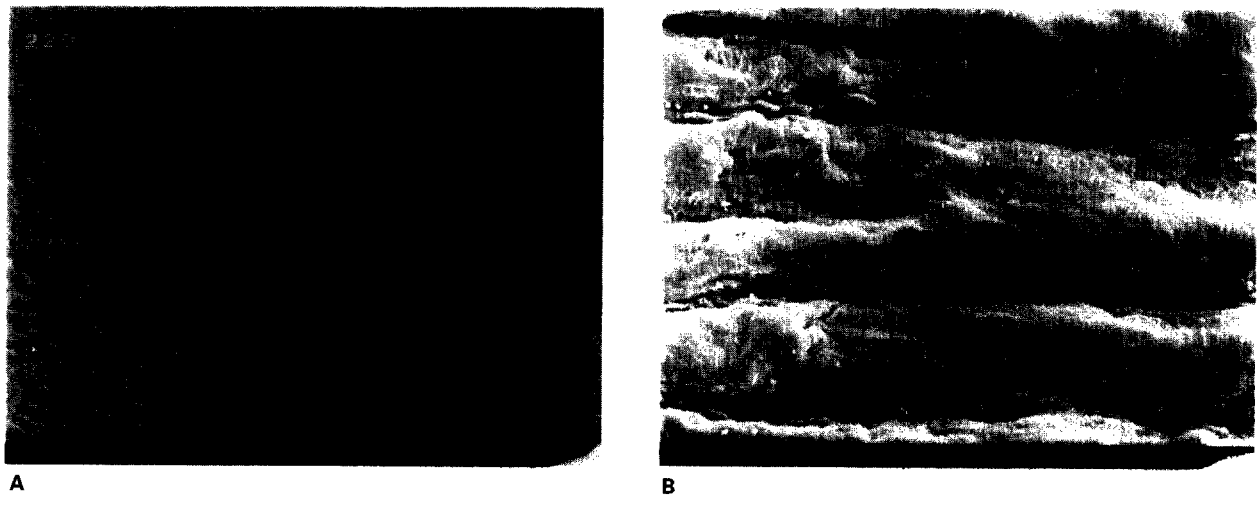
Scanning electron microscopy

75–90% of the endothelial cells were still present after the perfusion procedure. No difference was found between the different protocols studied. No platelet aggregates were present, but single scattered platelets were found (Fig. 7).

DISCUSSION

The decline in release of prostanoids with time, when vessels are being perfused, is in accordance with reports with perfused canine vessels (15–16), rabbit and rat aortas (23–25) and caval veins (23) as well as from perfused human umbilical veins (19). In the present model the vessels respond with increasing 6-keto-$PGF_{1\alpha}$ in response to AA, thrombin and bradykinin, given both to each perfusate as well as given after 60 min of perfusion. With addition of these substances, the same decline in PG release is seen, but at a higher level compared to HBSS perfusions (14). The mechanism for this decline is discussed elsewhere (14) and will not be repeated here, but it seems likely to be the result of an accommodation rather than a run out of substrate.

Papaverine prevented spasm in the vessels when given in vivo, but this administration of papaverine did not influence the prostacyclin release from arteries perfused ex vivo in the present study. It is therefore unlikely that the vasodilating effect of papaverine in arteries was mediated through the prostacyclin pathway in the endothelial cell. On the other hand, there was a lower initial release of prostacyclin from veins given papaverine prior to dissection. This could have been the effect of a more vasodilating effect on venous tissue than on arterial, maybe because of better penetration of papaverine through the thinner venous wall. This in turn would then lead to a lesser spasm during the operative procedure and lessened stimulation of the arachidonic pathway in the vascular wall. Another explanation may be that papaverine penetrated into the vessel wall and was released with the onset of vessel perfusion leading to interference with the assay. An experiment with ^{125}I would have given a definite answer to this question. When vessel segments are mounted in this perfusion setup, there is no vasoconstriction left, but we cannot rule out a certain amount of tension in the vessel wall. Data derived from cultured endothelial cells suggest papaverine to inhibit the arachidonic acid pathway at the phospholipase level (27). To study if the lessened release noted in the present study was a direct effect of papaverine on the endothelial cells, the next step was therefore to add papaverine directly to the perfusate. Using the ^{3}H 6-keto-$PGF_{1\alpha}$ RIA method almost no re-



A B

Fig. 7 Scanning electron microscopy of a perfused mongrel dog carotid artery. Most of the endothelial cells are present, but some are folded and disrupted. (A) Magnification 103 times. (B) Magnification 629 times.

lease was seen, when venous segments were perfused without or with AA. This did not correspond with the concept that papaverine may inhibit the prostanoid production at the phospholipase level, whereas the cyclooxygenase should be unaffected. Furthermore, the theory that papaverine should block the release of the most potent vasodilator known, as well as simultaneously prevent vasospasm, seemed unlikely. As the detectable amounts of 6-keto-$PGF_{1\alpha}$ and TXB_2 were almost abolished by papaverine when using ^3H RIA, a possible explanation might be that the assay was interfered with papaverine. A new radioimmunoassay with ^{125}I instead of ^3H tracer was therefore conducted where the pellet instead of the supernatant was counted for radioactivity. The ^{125}I assay displayed no difference in prostacyclin release between segments given papaverine to the perfusate and controls. The two assays use the same antibody and the same type of tracer but with different types of radioactivity. It is well known that the use of derivatized tracers in immunoassay can alter the specificity of a method even in assays using the same antibody (28). Thus, although the known cross-reaction with other prostaglandins in both assays was similar, the observed discrepancy between results by the ^3H- and ^{125}I-based methods might be evidence of a difference in the cross-reactivity of an unidentified prostaglandin by the two methods. However, in perfusate samples both with and without added papaverine, HPLC followed by ^3H RIA showed a single peak of immunoactivity which was associated with the elution time of 6-keto-$PGF_{1\alpha}$ standard. Surprisingly, the apparent level of 6-keto-$PGF_{1\alpha}$ in the perfusate with papaverine, which had previously (i.e. before HPLC) been very low by the ^3H-based assay, was now as high as with the ^{125}I-based method. This suggested that an interfering substance affecting only the ^3H-based assay was being removed from this sample by the HPLC purification. Indeed, the addition of papaverine (0.6 mg/ml) to the ^3H immunoassay was shown to reduce the efficiency of the charcoal suspension in adsorbing the tracer. This accounts for the artefactually lowered estimates of 6-keto-$PGF_{1\alpha}$ concentration in the presence of high levels of papaverine by the ^3H-based method.

The fact that the cyclooxygenase inhibitor diclofenac failed to prevent the dilatatory effect of papaverine supports the above finding that the effects of papaverine are not mediated through the arachidonic acid cascade. No explicit test of TXB_2 release using ^{125}I tracer was performed, but the effect of papaverine also on TXB_2 release makes it likely to be explained by the same mechanism.

In summary, this study first demonstrated that papaverine does not influence the release of prostacyclin in canine arteries and probably not in canine veins and that the vasodilating effect of papaverine is not mediated via the prostaglandin pathway. Second, it illustrates the sensitivity of the charcoal separation technique of immunoassays to non-specific interference.

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