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CYCLOOXYGENASE INVOLVEMENT IN THROMBOXANE-DEPENDENT CONTRACTION IN RAT MESENTERIC RESISTANCE ARTERIES

Manlio Bolla^{1*}, Dong You², Laurent Loufrani^{2§}, Bernard I Levy², Sylviane Levy-Toledano¹, Aïda Habib^{1#}, Daniel Henrion²

Institut National de la Santé et de la Recherche Médicale (INSERM) ¹Unit 348 and ²Unit 541, IFR Circulation-Paris-Nord, Paris, France.

Short title: PGE₂ mediated TxA₂-vasoconstriction

* current address: NicOx SA, 2455 route des Dolines, Sophia-Antipolis, France

current address : American University of Beirut, Dept of Biochem. and Int. Med., Beirut, Lebanon

§ current address: Dept of Physiology – CNRS UMR 6188, Medical School, Angers, France.

Corresponding author and present address:

Dr. Daniel HENRION, Pharm.D., Ph.D.

INSERM Unit 541, 41 bd de la chapelle

75475 PARIS, FRANCE

tel: 332 41 73 58 45

fax: 332 41 73 58 95

E-mail: daniel.henrion@med.univ-angers.fr

ABSTRACT

The influence of cyclooxygenase pathway activation following endoperoxide / thromboxane receptor (TP) stimulation was studied in rat mesenteric resistance arteries (n=6-10 per group). We studied isolated, perfused, and pressurized mesenteric resistance arteries (mean internal diameter 214 µm) using an arteriograph allowing to study arteries in physiological conditions of flow and pressure. Changes in diameter were continuously recorded and contractions were appreciated as internal diameter reduction. Release of cyclooxygenase pathway metabolites was also assessed by EIA analysis of mesenteric bed perfusions. The TxA₂ analog, U-46619 (1 µmol/L) induced a significant contraction (108 µm maximal diameter reduction). Inhibition by 3 chemically different cyclooxygenase inhibitors, flurbiprofen, indomethacin and aspirin, potently reduced the contraction to 27, 25 and 6% of control, respectively. The selective cyclooxygenase-1 inhibitor SC-58560 inhibited U-46619-contraction whereas selective cyclooxygenase-2 inhibition (SC-58236) had no effect. Thromboxane synthase inhibition (furegrelate) did not affect U-46619-induced contraction, but it was reduced by cytosolic phospholipase A2 inhibition. Measurement of cyclooxygenase derivatives produced by the isolated mesenteric bed showed that PGE₂ was produced after TxA₂-receptor stimulation with U-46619. Exogenous prostaglandin E₂ (in the presence of the TxA₂ receptor antagonist SQ 29,548) and U-46619 contracted mesenteric arteries with a similar potency (EC₅₀: 0.30 and 0.48 µmol/L, respectively). This study provides the first evidence that TxA₂-receptor-dependent contraction in a resistant artery involved cyclooxygenase- stimulation and, at least in part, PGE₂ formation. This mechanism of TxA₂-dependent contraction in resistant arteries might be of importance in the understanding of diseases affecting resistant arteries and involving TxA₂, such as hypertension.

INTRODUCTION

Thromboxane A₂ (TxA₂), a lipid mediator originating from arachidonic acid (AA) metabolism through the cyclooxygenase (COX) pathway, is a powerful constrictor of vascular smooth muscle (1). Enhanced TxA₂ production has been reported in several cardiovascular diseases such as unstable angina (2), acute myocardial infarction (2), spontaneous hypertension (3) and pregnancy-induced hypertension (4). In resistance arteries, TxA₂ is produced upon flow stimulation (shear stress) of the endothelium and this production is increased in the case of hypertension (5). Similarly, the involvement of TxA₂ also increases the pressure-induced myogenic tone in hypertension (6).

TxA₂ acts through specific G-protein coupled (1). Activation of TxA₂ receptor (TP; also known as thromboxane/endoperoxide receptor) leads to phospholipase C activation, release of inositol triphosphate (IP3), and increase in the intracellular Ca²⁺ level, thus triggering the smooth muscle contraction (7).

Cyclooxygenases are important enzymes in the formation of prostaglandins and TxA₂. They present a double enzymatic activity, cyclooxygenase before and peroxidase after, producing from the precursor AA, the unstable endoperoxide intermediates PGG₂ and PGH₂, respectively. PGH₂ itself already possesses activity (1,8), or alternatively it may be metabolized by different isomerases or synthases into prostaglandins, prostacycline (PGI₂) or TxA₂ (1,9). Two isoforms of COX are known; one constitutive (named COX-1) and the second one inducible (named COX-2) (10,11).

Resistance arteries are known to play an important role in the generation of peripheral vascular resistance, and are involved in the control of local blood distribution and capillary pressure (12, 13). Their vascular tone is regulated by the sympathetic nervous system, circulating hormones, and vasoactive metabolites produced by endothelial cells, among them TxA₂ (14). TxA₂ contributes to the homeostasis of normal resistance arteries, and an alteration of its synthesis and/or release was shown in pathological states, such as hypertension (5,6).

Involvement of COX in the vascular response of resistance arteries to different stimuli has been demonstrated. Vasoactive agents like angiotensin II (15), endothelin (16) and phenylephrine (17) have been shown to activate, and in part to induce contraction via, the COX pathway.

The aim of our work was to evaluate the influence of COX on the response of resistance arteries under conditions of regulated flow and pressure, following TP receptor stimulation by the stable agonist U46619, and to identify the cyclooxygenase metabolite important to the TP-dependent contraction.

METHODS

Mesenteric artery in vitro

Male Wistar Kyoto rats (WKY) (Iffa Credo, France), aged for 8-10 weeks were used. Rats were anesthetized with sodium pentobarbital (50 mg/Kg) and their gut excised. The investigation was in accordance with the European Community standards on the care and use of laboratory animals. A second order mesenteric artery ($214 \pm 13 \mu\text{m}$, internal diameter, 3-5 mm length) was cannulated at both ends and mounted in a video monitored perfusion system as previously described (17) according to Halpern *et al.* (18). The arterial segment was bathed in a Physiological Salt Solution (PSS) (17) and superfused (2 mL/min). Perfusion of the artery was set at a rate of 90 $\mu\text{L}/\text{min}$. Flow was set at 90 $\mu\text{l}/\text{min}$ and pressure at 50 mm Hg. Arterial diameter was measured and recorded continuously using a video monitoring system (Living System Instrumentation Inc). Data was collected (Biopac, La Jolla, CA, USA), recorded and analyzed (AcqKnowledge® software, Biopac) with the results given in μm for arterial inner diameters.

Vessels first were allowed to stabilize for at least 30 min. Vessels were then stimulated with 60 mmol/L KCl in order to verify vessel viability. Testing the vasodilator effect of acetylcholine (1 $\mu\text{mol}/\text{L}$) after precontraction with phenylephrine (1 $\mu\text{mol}/\text{L}$) then assessed the integrity of the endothelium. The response to U-46619 (0.01 to 10 $\mu\text{mol}/\text{L}$ or a single dose of 1 $\mu\text{mol}/\text{L}$), a selective and stable thromboxane receptor agonist, was assessed at least twice to obtain a stable and constant contraction. In some experiments, the preparation was preincubated with one of the following drugs: flurbiprofen, indomethacin, diclofenac and aspirin were used to inhibit COX activity (1), furegrelate to block TxA_2 synthesis (19), SC-58560 to inhibit COX-1, SC-58236 (20) to prevent COX-2 activation (20), SC-19220, EP1 receptor antagonist (21). Following pretreatment, contraction to U-46619 was repeated and compared to the previous stable contraction to U-46619. At the end of all experiments a contraction to 60 mmol/L KCl was performed, thus showing no change in vessel reactivity compared to the first one. In separate series of experiments, the effect of indomethacin and flurbiprofen was tested on phenylephrine (1 $\mu\text{mol}/\text{L}$), endothelin-1 (10 nmol/L), PGE_2 (1 $\mu\text{mol}/\text{L}$), $\text{PGF}_{2\alpha}$ (1 $\mu\text{mol}/\text{L}$). These latter agonists as well as U-46619 were added to the PSS so that the PSS superfusing and perfusing the arteries contained the concentration of drug indicated between the parenthesis.

Mesenteric bed perfusion

Mesenteric beds were prepared as previously described (22, 23). Briefly, the abdomen of anaesthetized rats was opened and the gut exposed. The superior mesenteric artery was separated from surrounding fat tissue in the region of the aorta. The rat was then sacrificed by exsanguination cutting the abdominal aorta. A polyethylene catheter (diameter 0.5 mm) was inserted distally into the artery at its origin from the aorta, and the catheter fixed with surgical thread. The intestine was separated from the mesentery and the cannulated mesentery perfused (2.0 mL/min, 37 °C). The perfusion pressure was monitored continuously (cf. upper paragraph).

After a 30 min. stabilization period the preparation was stimulated with 1 $\mu\text{mol}/\text{L}$ phenylephrine, followed by 1 $\mu\text{mol}/\text{L}$ Ach to check the integrity of the preparations, for vascular smooth muscle and endothelial responses.

The perfusate was collected for 5 min. (10 mL total volume) for the basal and different stimuli.

Perfusate extraction and EIA analysis

To 10 ml of perfusate samples 10 μ L of formic acid, 10,000 cpm of [3 H]-TxB₂ (NEN) for extraction recovery and 1 mL of methanol were added. After vortexing, samples were centrifugated at 1,200 x g at 4 °C for 10 min. Supernates were loaded onto C18 Bakerbond silica columns (3 mL), washed with 5 mL water and then eluted with 3 mL of methanol. Samples were dried under a vacuum and resuspended in 1 mL of EIA buffer (EIA buffer: Phosphate buffer 0.1 mol/L, pH 7.4 containing 0.15 mol/L NaCl, 1 mmol/L EDTA and 0.1 % bovine serum albumin). Radioactivity was counted in a 100 μ L aliquot for recovery and the sample frozen at -20 °C until measurement of prostanoids.

EIA analysis

Immunoenzyme analysis (EIA) of 6-keto-PGF_{1 α} , the breakdown product of PGI₂, as well as PGE₂ and PGF_{2 α} were performed as previously described (24).

Statistical analysis

Results are expressed as means \pm s.e. mean of *n* measurements. The significance of the different treatments was determined by ANOVA or two tailed Student's paired T-test. P values less than 0.05 were considered to be significant.

Drugs

SC-58560 and SC-58236 were kindly provided by Dr Peter Isakson (Searle Monsanto, St Louis, USA). HEPES, acetylcholine (ACh), phenylephrine (Phe), acetylsalicylic acid (ASA), indomethacin and flurbiprofen, were purchased from Sigma Chem. Co. (St Louis, MO). Methyl arachidonylfluorophosphate (MAFP) was purchased from Calbiochem. Other reagents were purchased from Prolabo (Paris, France). Other prostanoids were purchased from Cayman Chem. Co. (Ann Arbor, MI).

RESULTS

Isolated mesenteric arteries

Perfused and pressurized rat mesenteric arteries responded to TP receptor stimulation with U-46619 by a concentration-dependent contraction ($EC_{50} = 0.48 \pm 0.06 \mu$ M, maximal response: $108 \pm 8 \mu$ m decrease in diameter, *n*=10). Pretreatment with 3 chemically non-related non-steroidal anti-inflammatory drugs (NSAIDs), flurbiprofen (1 μ mol/L), indomethacin (1 μ mol/L), and aspirin (10 μ mol/L) inhibited U-46619 (1 μ mol/L)-induced contraction to 27 ± 6 , 25 ± 5 and $6 \pm 3\%$ of control, respectively (Fig 1). Indomethacin (1 μ mol/L) and flurbiprofen (1 μ mol/L) also inhibited, at least in part, phenylephrine (1 μ mol/L) and endothelin-1 (10 nmol/L)-induced contraction (fig 1C). PGE₂ (1 μ mol/L)-induced contraction was also inhibited by aspirin (10 μ mol/L, $79 \pm 7\%$ of control, *n*=4), indomethacin (1 μ mol/L, $84 \pm 8\%$ of control, *n*=4), and flurbiprofen (1 μ mol/L, $84 \pm 9\%$ of control, *n*=4). PGF_{2 α} (1 μ mol/L)-induced contraction was inhibited by aspirin, (10 μ mol/L, $68 \pm 7\%$ of control, *n*=5), indomethacin (1 μ mol/L, $78 \pm 9\%$ of control, *n*=4), but not by flurbiprofen (1 μ mol/L, $94 \pm 8\%$ of control, *n*=5).

Removing the endothelium did not affect the inhibitory effect of indomethacin (1 $\mu\text{mol/L}$) on U-46619-induced contraction ($108 \pm 9 \mu\text{m}$ decrease in diameter versus 18 ± 4 ; $n=5$).

The selective COX-1 inhibitor SC-58560 concentration dependently decreased CCRC to U-46619, whereas the COX-2 selective inhibitor (SC-58236) was mainly ineffective (Fig 2).

Phospholipase A2 (PLA2) inhibition with MAFP (10 $\mu\text{mol/L}$) reduced to 13% of the control the maximal response to U-46619 ($20 \pm 5 \mu\text{m}$ loss in diameter after MAFP versus $155 \pm 15 \mu\text{m}$ in control, $n=6$). This inhibition completely overlapped inhibition given by the NSAIDs. By contrast, Phe (1 $\mu\text{mol/L}$)-induced contraction was only reduced to $54 \pm 6\%$ ($n=4$) of the control by MAFP (10 $\mu\text{mol/L}$).

Thromboxane synthase inhibition (furegrelate, 10 $\mu\text{mol/L}$) left the maximal response unchanged to U-46619 ($84 \pm 9 \mu\text{m}$ contraction in the presence of furegrelate versus $89 \pm 10 \mu\text{m}$ in control, $n=6$). On the other hand, Phe (1 $\mu\text{mol/L}$) and endothelin-1 (10 nmol/L)-induced contraction were decreased, respectively, to $46 \pm 7\%$ ($n=4$) and $41 \pm 7\%$ of control by furegrelate (10 $\mu\text{mol/L}$).

$\text{PGF}_{2\alpha}$ and PGE_2 are 2 other possible COX-derived compounds that might be involved in U-46619-induced contraction in MRA. CCRC to exogenous $\text{PGF}_{2\alpha}$ showed a contractile response only at high concentrations (fig.3). The response to $\text{PGF}_{2\alpha}$ (10 $\mu\text{mol/L}$) was abolished by the TP receptor antagonist SQ 29,548, 10 $\mu\text{mol/L}$ (Fig. 4).

The second exogenous metabolite tested was PGE_2 (fig.3). The contraction elicited by PGE_2 (1 $\mu\text{mol/L}$) was not significantly affected by SQ 29,548 (fig.4), suggesting a direct contractile action of PGE_2 .

The TP receptor antagonist SQ 29,548 induced no significant inhibition of phenylephrine-induced contraction (data not shown), but it induced a $34 \pm 5\%$ ($n=5$) inhibition of endothelin-1-induced tone.

The EP1 receptor antagonist SC-19220 (10 $\mu\text{mol/L}$) significantly inhibited U-46619-induced contraction to a level equivalent to 48% of control (94 ± 7 versus $45 \pm 9 \mu\text{m}$ decrease in diameter, $n=6$). On the other hand, ET-1-induced contraction was not significantly reduced by SC-19220 (105 ± 17 versus $88 \pm 12 \mu\text{m}$ decrease in diameter, $n=4$).

In the absence of endothelium SC-19220 (10 $\mu\text{mol/L}$) inhibited similarly U-46619-induced contraction ($112 \pm 10 \mu\text{m}$ decrease in diameter versus 61 ± 7 ; $n=5$).

EIA analysis of perfused mesenteric beds

An immunometric measurement for 6-keto- $\text{PGF}_{1\alpha}$ (the stable metabolite of PGI_2), $\text{PGF}_{2\alpha}$ and PGE_2 was performed in the perfusate of isolated mesenteric beds. Unfortunately, U-46619 interferes with the dosage of 6-keto- $\text{PGF}_{1\alpha}$ and $\text{PGF}_{2\alpha}$ and the amount of U-46619 present in the PSS collected from perfused mesenteric beds made it impossible to determine this metabolite. Nevertheless, PGI_2 is more likely to induce dilation in mesenteric resistance arteries (5). However, a rise in 6-keto- $\text{PGF}_{1\alpha}$ immunoreactivity was shown after of cholinergic receptor stimulation with acetylcholine, confirming that the endothelial layer was intact. A second confirmation was recognized in the fall of perfusion pressure from $81 \pm 6 \text{ mmHg}$ to $32 \pm 5 \text{ mmHg}$ in the presence of acetylcholine after phenylephrine-induced precontraction ($n=6$).

On the contrary, a two-fold rise in PGE_2 secretion was observed in the presence of U-46619 stimulation (Fig. 5), with a very low (less than 1% of the final amount calculated) interference of U-46619 in assaying PGE_2 .

Discussion

The present study brings new insights in the comprehension of the physiological role of thromboxane A₂ (TxA₂) in resistance arteries. Surprisingly, contractions induced by the stable TP receptor agonist U-46619 were strongly attenuated by non-selective COX inhibitors and by a selective COX-1 inhibitor. A further analysis showed that PGE₂ may be, at least in part, the mediator involved in U-46619-induced contraction in rat mesenteric resistance arteries.

Resistance arteries determine vascular resistance and hence have a key role in the control of local blood flow (13). There are an increasing number of studies showing that TxA₂ may have a significant role in the vascular homeostasis in several vascular beds (3,5,6). This involvement of TxA₂ in the control of vascular tone is enhanced in pathological situations such as hypertension (3-6). In addition, we have shown in resistance arteries, that TP receptor activation does not desensitize (17). Our finding that U-46619-induced contraction was almost abolished by COX inhibition is surprising as TxA₂ production itself depends on COX activation. COX derivatives have been involved in agonist-induced contraction due to angiotensin II (15), endothelin-1 (16) or alpha-adrenoreceptor stimulation (17). In most cases the mediator involved is probably TxA₂ and/or PGH₂ as contraction was blocked by COX inhibition and by a TP receptor antagonist. In the present study, TP receptor activation by U-46619 also depended on COX derivatives to produce a contraction. This was confirmed by the use of 3 non-selective and chemically different cyclooxygenase inhibitors (aspirin, flurbiprofen and indometacin) and by the use of a selective COX-1 inhibitor (SC-58560). COX-2 inhibition was ineffective. In addition, PLA2 inhibition also prevented U-46619-induced contraction, and thusly further supporting the involvement of a COX and arachidonic acid-derivative.

Among the COX-derivatives that may be produced in resistance arteries, TxA₂, PGF_{2 α} and PGE₂ are the main vasoconstrictor products. In the arteries used in the present study, PGF_{2 α} was not potent enough to be involved in U-46619-induced contraction as it only induced a contraction for concentrations higher than 1 μ mol/L, possibly interacting with TP receptor (twice the EC₅₀ for U-46619). On the other hand, PGE₂ produced a contraction comparable to that of U-46619. PGE₂ has been shown to enhance vasoconstrictor responses to pressure stimuli in the rat mesenteric artery (24). It is also a potent vasoconstrictor in the rat tail artery (25) and the aorta (26). We performed an immuno-enzymatic assay of PGE₂ in mesenteric arterial beds perfused with U-46619 in order to further investigate the potential involvement of PGE₂ in TP receptor activation-dependent contraction. Under U-46619 stimulation, the PSS perfused in the mesenteric beds contained twice the baseline PGE₂ concentration, further supporting that PGE₂ may be involved in the contraction induced by TP receptors stimulation. The technique used to measure COX-derivatives in perfused mesenteric arteries has been previously validated in a study showing that more TxA₂ and less PGI₂ were produced in the PSS flowing through the arterial bed from hypertensive rats, compared to normotensive animals (5). In this latter study we have shown that 6-keto-PGF_{1 α} (1.5 ng/mL, the stable metabolite of PGI₂), PGF_{2 α} (0.6 ng/mL) and TxB₂ (1.5 ng/mL, stable metabolite of TxA₂) were also produced by the mesenteric circulation in the absence of exogenous stimulation. The amount of PGI₂, PGF_{2 α} and TxA₂ previously found (5) is comparable to the amount of PGE₂ found in the present study (1-2 ng/mL).

Interactions between different pathways may also occur. Although COX inhibitors almost completely inhibited U-46619-dependent contraction, they also partially inhibited the contraction induced by

endothelin-1 and phenylephrine, without affecting PGE₂-induced tone. In parallel, TP receptor blockade, which totally inhibited U46169- and PGF_{2α}-induced contractions, also inhibited endothelin-1 and phenylephrine-dependent tone to a lesser extent. Indeed, the contractile effect of these vasoactive agents (U46169, endothelin-1 and phenylephrine) depends on the activation of PLA2 induced by the rise in calcium concentration following receptor activation (1). Then, arachidonic acid release and COX activation lead to the production of different COX-derivatives. Concerning U-46619 in mesenteric resistance arteries, COX activation induced the production of PGE₂, which was responsible for a large part of the contraction. On the other hand, endothelin-1-induced contraction, also inhibited in a great part by PLA2 and COX antagonists, was strongly reduced by TP receptor blockade and unaffected by EP1 inhibition. Thus, TP receptor activation by TxA₂/PGH₂ is involved in ET-1 induced contraction, not that to PGE₂. This also applies to Phe-induced contraction, but to a lesser extent (PLA2 and COX inhibition only reduced Phe-induced contraction by 50%). Thus, the different contractile agents studied involved COX derivatives, but to a different degree. In addition, they required either PGE₂ or TxA₂/PGH₂ to produce a large part of their contractile effect.

Perspective:

This finding might be of importance in the pathophysiology of vascular diseases such as hypertension. In hypertension, TxA₂ has been shown to be involved in the control of vascular tone in arterial beds where it is normally (in the normotensive state) not involved or involved to a lesser degree. For example, a higher TxA₂ production in response to shear stress is likely to reduce flow-mediated dilation in mesenteric resistance arteries (5). Similarly, in rat gracilis muscle, resistance arteries TxA₂ contributes to the elevation in myogenic tone found in SHR (6). Thus, elevated TxA₂-production may contribute to the changes in local blood flow found in hypertension, thereby, participating to the injury produced in end-organs.

Considering the importance of TxA₂ in the local control of vascular tone, at least in some arterial beds, the present finding may enhance our understanding of vascular diseases as well as assist in focusing on potential treatments. In that respect, it may be important to decrease the vasoconstrictor capacity of TxA₂ without affecting its other properties. The potential importance of TP receptor blockade in other diseases has been recently discussed in a review (27), the issue being of importance in atherogenesis (27, 28).

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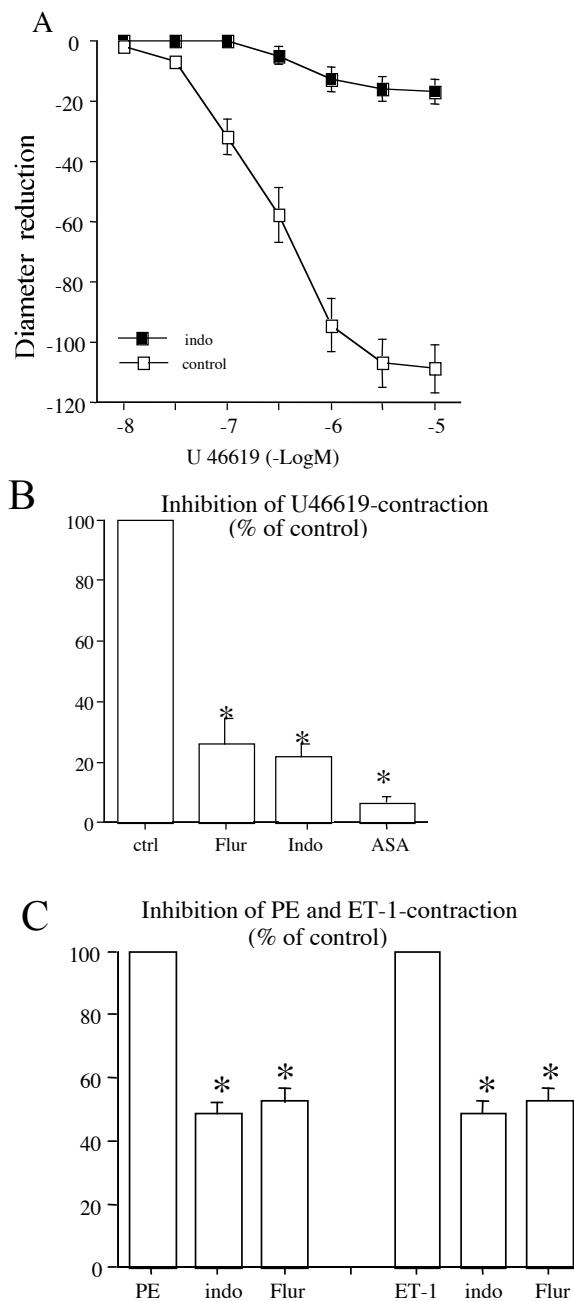


Figure 1: A: Concentration-response curves to U-46619 obtained in isolated perfused and pressurized rat mesenteric arteries. Concentration-response curves to U-46619 were repeated in the presence of the non-selective COX inhibitor indomethacin (**indo** 1 $\mu\text{mol/L}$, n = 6 per group).

B: Inhibitory effect of three chemically non-related non-steroidal anti-inflammatory drugs, indomethacin (1 $\mu\text{mol/L}$, **Indo**), flurbiprofen (1 $\mu\text{mol/L}$, **Flur**) and aspirin (10 $\mu\text{mol/L}$, **ASA**) on U-46619 (1 $\mu\text{mol/L}$)-induced contraction.

C: Inhibitory effect of indomethacin (1 $\mu\text{mol/L}$, **Indo**) and flurbiprofen (1 $\mu\text{mol/L}$, **Flur**) on phenylephrine (Phe, 1 $\mu\text{mol/L}$)- and endothelin-1 (ET-1, 10 nmol/L)-induced contraction.

Contraction was obtained in perfused and pressurized rat mesenteric resistance arteries (n=10 per group). *P < 0.05; two tailed Student's paired T-test.

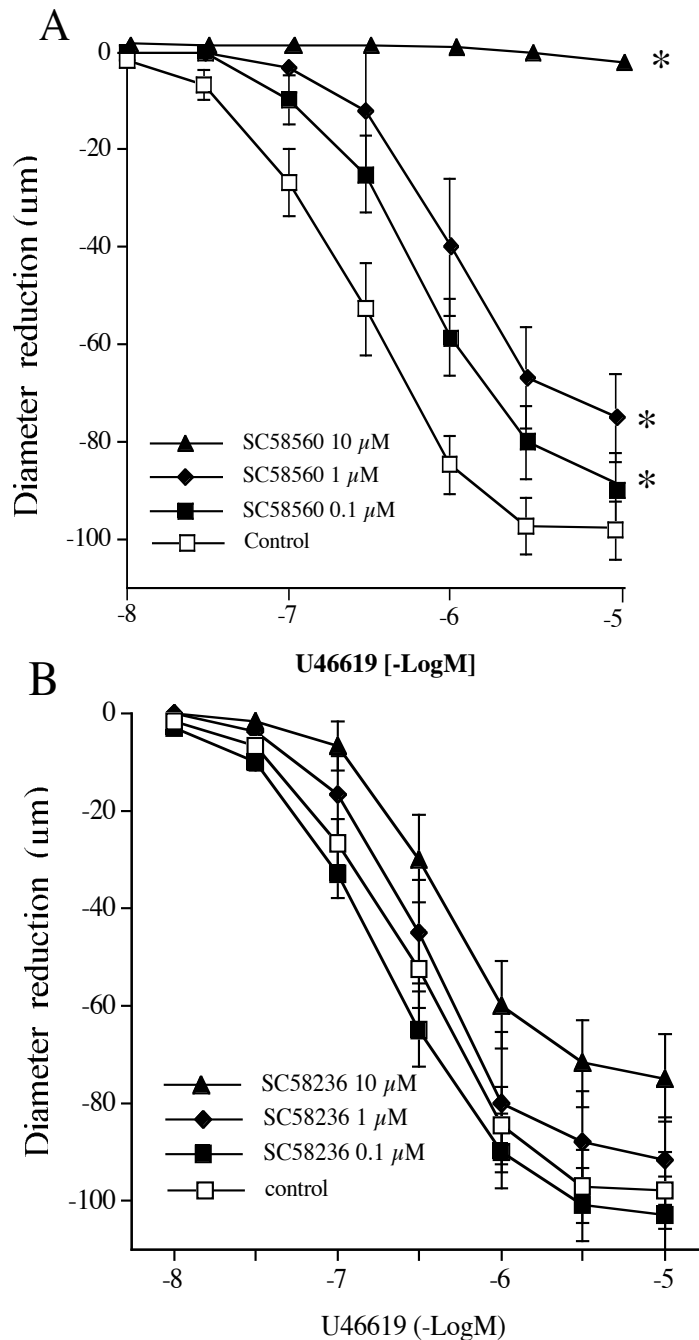


Figure 2: Changes in diameter in response to increasing concentrations of the TP receptor agonist U-46619, in perfused and pressurized rat mesenteric resistance arteries (n=8 per group, mean \pm sem). Concentration-response curves to U-46619 were repeated in the presence of the selective COX-1 inhibitor SC-58560 (upper panel, **A**) or in the presence of the COX-2 selective inhibitor (SC-58236) (lower panel, **B**). Control experiments were conducted in the presence of the solvent (<0.1% DMSO).

*P < 0.05; two-factor ANOVA.

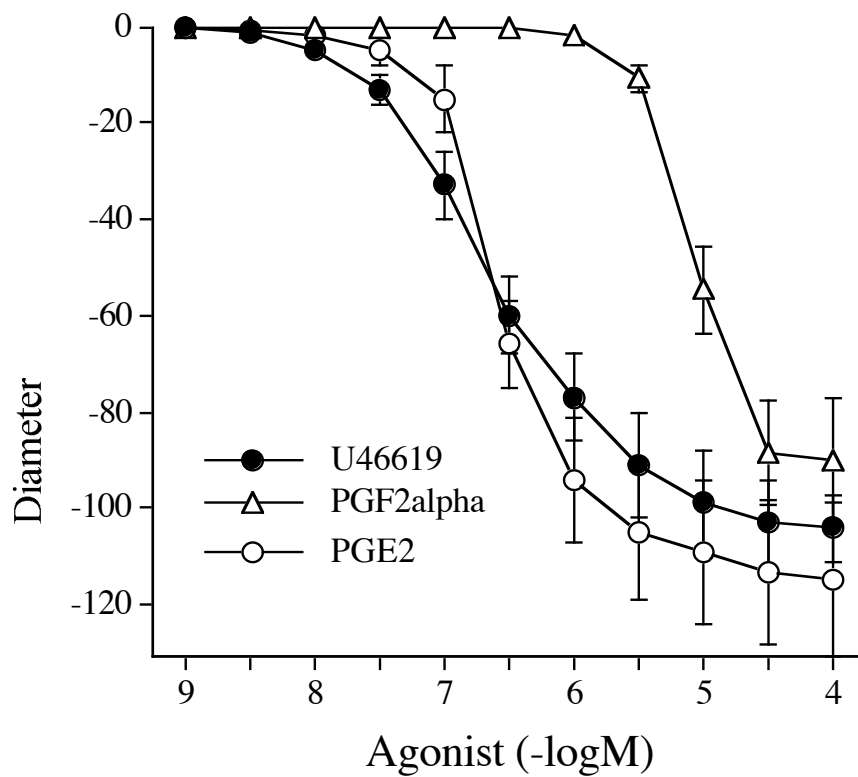


Figure 3: Changes in diameter in response to increasing concentrations (1 nmol/L to 0.1 μ mol/L) of the TP receptor agonist U-46619, PGF_{2 α} or PGE₂ in perfused and pressurized rat mesenteric resistance arteries (n=8 per group, mean \pm s.e.m.).

*P <0.05; two tailed Student's T-test.

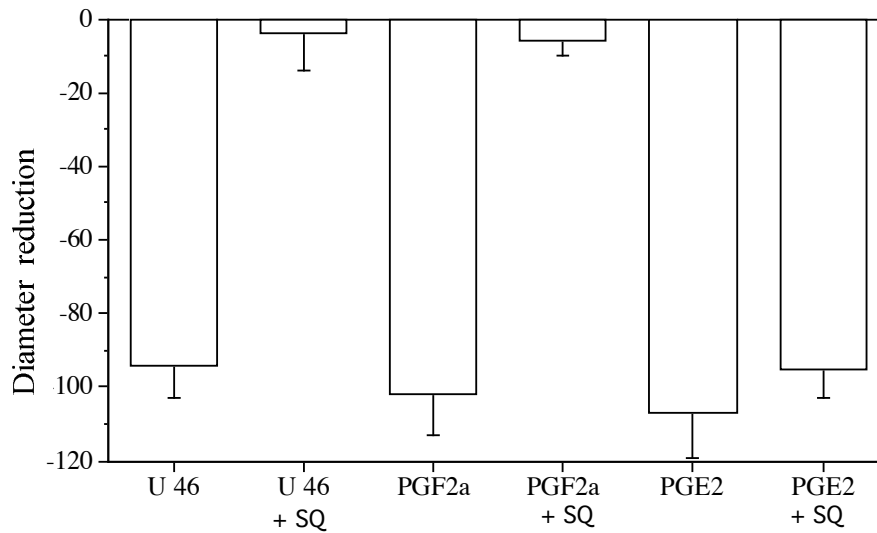


Figure 4: Inhibitory effect of the TP receptor antagonist SQ 29,548 (SQ, 10 $\mu\text{mol/L}$) on U-46619 (U46, 1 $\mu\text{mol/L}$)-, $\text{PGF}_{2\alpha}$ (10 $\mu\text{mol/L}$)- or PGE_2 (1 $\mu\text{mol/L}$)-induced contraction. Contraction was obtained in perfused and pressurized rat mesenteric resistance arteries (n=8 per group).

*P <0.05; two tailed Student's T-test.

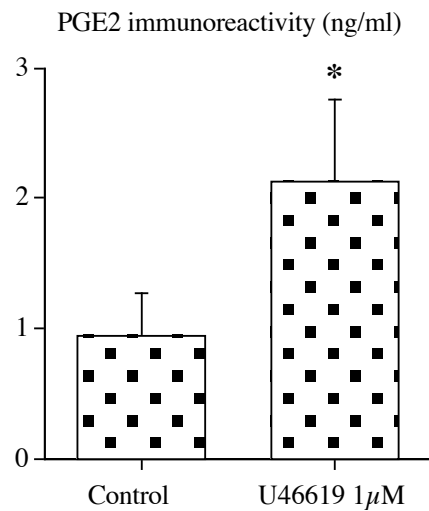


Figure 5: Immunometric analysis of PGE_2 were done using specific antibodies and tracers in the presence or in the absence (control) of U-46619, in the PSS collected from rat perfused mesenteric beds.

*P <0.05; two tailed Student's T-test.