Cyclooxygenase Involvement in Thromboxane-Dependent Contraction in Rat Mesenteric Resistance Arteries

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Abstract—The influence of cyclooxygenase pathway activation following thromboxane-endoperoxide (TP) receptor stimulation was studied in rat mesenteric resistance arteries (n=6 to 10 per group). We studied isolated, perfused, and pressurized mesenteric resistance arteries (mean internal diameter 214 μm) using an arteriograph, enabling us to study arteries in physiological conditions of flow and pressure. Changes in diameter were continuously recorded, and contractions measured as internal diameter reduction. Release of cyclooxygenase pathway metabolites was also assessed by enzyme immunoassay (EIA) analysis of mesenteric bed perfusions. The thromboxane A2 (TxA2) analog U-46619 (1 μmol/L) induced a significant contraction (108 μm maximal diameter reduction). Inhibition by 3 chemically different cyclooxygenase inhibitors (ie, 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, a 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. (Hypertension. 2004;43:1264-1269.)

Key Words: resistance ■ cyclooxygenase ■ prostaglandins ■ thromboxane

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 on 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 triphosphate1 (IP3), and an 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 TxA2.9 Two isoforms of COX are known: one constitutive (COX-1); the second one inducible (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 vasomotor metabolites produced by endothelial cells, among them TxA2.14 TxA2 contributes to the homeostasis of normal resistance arteries, and an alteration of its synthesis 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. Vasowac-
tive agents like angiotensin II,15 endothelin,16 and phenylephrine17 have been shown to activate and, in part, to induce contraction through 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 COX metabolite important to the TP-dependent contraction.

Methods

Mesenteric Artery in Vitro

Male Wystar Kyoto rats (WKY; Ifla Credo, France), aged 8 to 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 ± 13 μm, internal diameter, 3 to 5 mm length) was cannulated at both ends and mounted in a video-monitored perfusion system as previously described17 and according to Halpern and Kelley.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 μL/min; flow was set at 90 μL/min and pressure at 50 mm Hg. Arterial diameter was measured and recorded continuously using a video monitoring system (Living System Instrumentation Inc). Data were collected, recorded, and analyzed (AcqKnowledge software, Biopac) with the results given in micrometers for arterial inner diameters.

Vessels first were allowed to stabilize for at least 30 minutes and were then stimulated with 60 mM/L KCl to verify vessel viability. Next, endothelium integrity was assessed by testing the vasodilator effect of acetylcholine (Ach; 1 μmol/L) after preconstriction with phenylephrine (Phe; 1 μmol/L). The response to U-46619 (0.01 to 10 μmol/L or a single dose of 1 μmol/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 (used to inhibit COX activity)19; furegrelate (to block TxA2 synthesis)19; SC-58560 (to inhibit COX-1); SC-5823620 (to prevent COX-2 activation)20; or SC-19220 (an EP1 receptor antagonist).21 After pretreatment, contraction to U-46619 was repeated and compared with the previous stable contraction to U-46619. At the end of all experiments, a contraction to 60 mM/L KCl was performed, thus showing no change in vessel reactivity compared with the first. In a separate series of experiments, the effect of indomethacin and flurbiprofen was tested on phenylephrine (1 μmol/L), endothelin-1 (10 μmol/L), PGE2 (1 μmol/L), and PGF2α (1 μmol/L). These latter agonists as well as U-46619 were added to PSS so that the PSS superfusing and perfusing the arteries contained the concentration of each drug, as indicated in parentheses above.

Mesenteric Bed Perfusion

Mesenteric beds were prepared as previously described.22,23 Briefly, the abdomen of anesthetized 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 euthanized 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 mesenteric perfused (2.0 mL/min, 37°C). The perfusion pressure was monitored continuously (see Mesenteric Artery In Vitro).

After a 30-minute stabilization period, the preparation was stimulated with 1 μmol/L Phe, followed by 1 μmol/L Ach to check the integrity of the preparations for vascular smooth muscle and endothelial responses. The perfusate was collected for 5 minutes. (10 mL total volume) for the basal and different stimuli.

Perfusate Extraction and Enzyme Immunoassay Analysis

To 10 mL of perfusate samples and 10 μL of formic acid were added 10 000 cpm of [3H]TXB2 (NEN) for extraction recovery and 1 mL of methanol. After vortexing, samples were centrifuged at 1200g at 4°C for 10 minutes. 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 enzyme immunoassay (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 μL aliquot for recovery and the sample frozen at −20°C until measurement of prostanoids.

EIA Analysis

EIA of 6-keto-PGF1α, the breakdown product of PGI2 as well as PGE2 and PGF2α, was performed as previously described.24

Statistical Analysis

Results are expressed as means±SEM of n measurements. The significance of the different treatments was determined by ANOVA or 2-tailed Student paired t test. P<0.05 was considered significant.

Drugs

SC-58560 and SC-58236 were kindly provided by Dr Peter Isakson (Searle Monsanto, St Louis, Mo). HEPES, Ach, Phe, acetylsalicylic acid (ASA), indomethacin, and flurbiprofen were purchased from Sigma. Methyl arachidonylfluorophosphate (MAFP) was purchased from Calbiochem. Other reagents were purchased from Prolabo (Paris, France). Other prostanoids were purchased from Cayman Chemical (Ann Arbor, Mich).

Results

Isolated Mesenteric Arteries

Perfused and pressurized rat mesenteric arteries responded to TP receptor stimulation with U-46619 by a concentration-dependent contraction (EC50=0.48±0.06 μmol/L, maximal response: 108±8 μm decrease in diameter, n=10). Pretreatment with 3 chemically nonrelated nonsteroidal anti-inflammatory drugs (NSAIDs), flurbiprofen (1 μmol/L), indomethacin (1 μmol/L), and aspirin (10 μmol/L) inhibited U-46619–induced (1 μmol/L) contraction to 27±6, 25±5, and 6±3% of control, respectively (Figure 1). Indomethacin (1 μmol/L) and flurbiprofen (1 μmol/L) also inhibited, at least in part, Phe (1 μmol/L) and endothelin-1–induced (10 μmol/L) contraction (Figure 1C). PGE2-induced (1 μmol/L) contraction was also inhibited by aspirin (10 μmol/L, 79±7% of control, n=4), and indomethacin (1 μmol/L, 84±8% of control, n=4), and flurbiprofen (1 μmol/L, 84±9% of control, n=4). PGE2α-induced (1 μmol/L) contraction was inhibited by aspirin (10 μmol/L, 68±7% of control, n=5) and indomethacin (1 μmol/L, 78±9% of control, n=4), but not by flurbiprofen (1 μmol/L, 94±8% of control, n=5).

Removing the endothelium did not affect the inhibitory effect of indomethacin (1 μmol/L) on U-46619–induced contraction (108±9 μm decrease in diameter versus 18±4, n=5). The selective COX-1 inhibitor SC-58560 concentration dependently decreased concentration-response curve (CRC) to U-46619, whereas the COX-2 selective inhibitor (SC-58236) was mainly ineffective (Figure 2).

Phospholipase A2 (PLA2) inhibition with MAFP (10 μmol/L) reduced to 13% of the control the maximal response to U-46619 (20±5 μm loss in diameter after MAFP.
versus 155±15 μm in control, n=6). This inhibition completely overlapped inhibition given by the NSAIDS. By contrast, Phe-induced (1 μmol/L) contraction was only reduced to 54±6% (n=4) of the control by MAFP (10 μmol/L).

Thromboxane synthase inhibition (furegrelate, 10 μmol/L) left the maximal response unchanged to U-46619 (84±9 μm contraction in the presence of furegrelate versus 89±10 μm in control, n=6). On the other hand, Phe (1 μmol/L) and endothelin-1--induced (10 nmol/L) contraction were de-
increased, respectively, to 46±7% (n=4) and 41±7% of control by furegrelate (10 μmol/L).

PGF_2α and PGE_2 are 2 other possible COX-derived compounds that might be involved in U-46619–induced contraction in mesenteric resistance arteries. CRC to exogenous PGF_2α showed a contractile response only at high concentrations (Figure 3). The response to PGF_2α (10 μmol/L) was abolished by the TP receptor antagonist SQ 29,548 (Figure 4).

The second exogenous metabolite tested was PGE_2 (Figure 3). The contraction elicited by PGE_2 (1 μmol/L) was not significantly affected by SQ 29,548 (Figure 4), suggesting a direct contractile action of PGE_2. The TP receptor antagonist SQ 29,548 induced no significant inhibition of Phe-induced contraction (data not shown), but it induced a 34±5% (n=5) inhibition of endothelin-1–induced tone.

The EP1 receptor antagonist SC-19220 (10 μmol/L) significantly inhibited U-46619–induced contraction to a level equivalent to 48% of control (94±7 versus 45±9 μm decrease in diameter, n=6). On the other hand, endothelin-1–induced contraction was not significantly reduced by SC-19220 (105±17 versus 88±12 μm decrease in diameter, n=4). In the absence of endothelium, SC-19220 (10 μmol/L) inhibited a similarly U-46619–induced contraction (112±10 μm decrease in diameter versus 61±7, n=5).

EIA Analysis of Perfused Mesenteric Beds
An immunometric measurement for 6-keto-PGF_1α (the stable metabolite of PGI_2), PGF_2α, and PGE_2 was performed in the perfusate of isolated mesenteric beds. Unfortunately, U-46619 interferes with the dosage of 6-keto-PGF_1α, and PGF_2α 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. However, a rise in 6-keto-PGF_1α immunoreactivity was shown after cholinergic receptor stimulation with Ach, confirming that the endothelial layer was intact. A second confirmation was recognized in the fall of perfusion pressure from 81±6 mm Hg to 32±5 mm Hg in the presence of acetylcholine after phenylephrine-induced precontraction (n=6). On the contrary, a 2-fold rise in PGE_2 secretion was observed in the presence of U-46619 stimulation (Figure 5), with a very low (<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 TXA_2 in resistance arteries. Surprisingly, contractions induced by the stable TP receptor agonist U-46619 were strongly attenuated by nonselective COX inhibitors and by a selective COX-1 inhibitor. Further analysis showed that PGE_2 may be, at least in part, the mediator involved in U-46619–induced contraction in rat mesenteric resistance arteries.
PLA2 inhibition also prevented U-46619 (SC-58560); COX-2 inhibition was ineffective. In addition, indometacin) and by the use of a selective COX-1 inhibitor was confirmed by the use of 3 nonselective and chemically 


dependent contraction. Under U-46619 stimulation, the PSS perfused in the mesenteric beds contained twice the baseline PGE2 concentration, further support 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 with normotensive animals. In this latter study, we have shown that 6-keto-PGFα (1.5 ng/mL, the stable metabolite of PGI2), PGFα (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, PGFα, and TxA2 previously found is comparable to the amount of PGE2 found in the present study (1 to 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 Phe, without affecting PGE2-induced tone. In parallel, TP receptor blockade, which totally inhibited U46169- and PGFα-α-induced contractions, also inhibited endothelin-1 and Phe-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. 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 EPI inhibition. Thus, TP receptor activation by TXA2/PGH2 is involved in endothelin-1-induced contraction, not in induced contraction due 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.

**Perspectives**

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. Similarly, in rat gracilis muscle, resistance arteries TxA₂ contribute to the elevation in myogenic tone found in spontaneously hypertensive rats. Thus, elevated TxA₂ production may contribute to the changes in local blood flow found in hypertension, thereby, contributing 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, the issue of importance in atherogenesis.

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