

Cyclooxygenase-1, not cyclooxygenase-2, is responsible for physiological production of prostacyclin in the cardiovascular system

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Prostacyclin is an antithrombotic hormone produced by the endothelium, whose production is dependent on cyclooxygenase (COX) enzymes of which two isoforms exist. It is widely believed that COX-2 drives prostacyclin production and that this explains the cardiovascular toxicity associated with COX-2 inhibition, yet the evidence for this relies on indirect evidence from urinary metabolites. Here we have used a range of experimental approaches to explore which isoform drives the production of prostacyclin in vitro and in vivo. Our data show unequivocally that under physiological conditions it is COX-1 and not COX-2 that drives prostacyclin production in the cardiovascular system, and that urinary metabolites do not reflect prostacyclin production in the systemic circulation. With the idea that COX-2 in endothelium drives prostacyclin production in healthy individuals removed, we must seek new answers to why COX-2 inhibitors increase the risk of cardiovascular events to move forward with drug discovery and to enable more informed prescribing advice.

nonsteroidal anti-inflammatory drugs | thrombosis |
COX-2 selective drugs | rofecoxib

Prostacyclin is an antithrombotic hormone produced by the vascular wall, inhibition of which has been associated with an increased risk of heart attacks and strokes (1–3). The production of prostacyclin is dependent upon cyclooxygenase (COX) enzymes, which convert arachidonic acid into prostaglandin H₂, the precursor of all prostanoids. Twenty years ago it was established that COX exists in two isoforms, COX-1 and COX-2 (4–6). Originally it was thought that COX-2 was largely responsible for the pathological production of prostanoids, for instance in inflammatory conditions, but now it is understood that COX-2 may fulfill physiological as well as pathological roles (7, 8). With regard to the cardiovascular system, there has been strong debate as to which isoform supports the vascular production of prostacyclin and it is currently widely believed that COX-2 and not COX-1 is expressed within endothelial cells (1, 2, 9, 10). Inhibition of COX isoforms explains both the therapeutic and deleterious effects of traditional nonsteroid anti-inflammatory drugs (NSAIDs; e.g., ibuprofen and diclofenac) as well as COX-2-selective drugs (e.g., celecoxib and rofecoxib). As it is commonly believed that COX-2 in endothelial cells is responsible for prostacyclin release, it is also thought that inhibition of endothelial COX-2 explains the increased incidence of atherothrombotic events that have been associated with the use of some traditional NSAIDs and COX-2-selective drugs (11, 12).

The idea that COX-2 drives vascular prostacyclin production is rooted particularly in studies showing that the urinary prostacyclin marker, 2,3-dinor-6-keto-PGF_{1α} (PGI-M) is reduced by COX-2-selective inhibitors (13, 14). Evidence from other experimental approaches, especially immunohistochemistry, has

in many regards failed to support this conclusion (15–19). The most recent study of vascular-targeted COX-2 gene deletion, for instance, reporting COX protein expression in cells in culture but not within the intact vasculature (10). Here we have used a range of experimental approaches including human cells, genetically modified laboratory animals where the COX-1 or COX-2 gene has been deleted, and measures of prostacyclin release in vitro and in vivo to explore which isoform drives the production of prostacyclin. Our data show that under physiological conditions it is COX-1 and not COX-2 that drives prostacyclin production in the cardiovascular system and that urinary measures of prostacyclin do not reflect endogenous prostacyclin production in the systemic circulation. The belief that COX-2 drives prostacyclin has had a major impact on the impetus to develop new therapeutic avenues and/or new drugs in the COX-2 inhibitor class. Thus, it is essential that we fully understand the role of COX-2 in the cardiovascular system to move forward with drug discovery and to enable more informed prescribing advice.

Results and Discussion

COX Expression and Activity by Cells in Vitro and Arteries ex Vivo. We have previously shown that COX-1, and not COX-2, is expressed in human aortic endothelial cells grown in culture under static conditions (15, 20). Endothelial cells grown under static conditions often quickly lose phenotypic markers. In particular, it has been suggested that COX-2 expression is lost rapidly in endothelial cells in static culture but that this can be rescued by applying shear stress (21–24). This conclusion regarding the regulation of expression of COX-2 by shear, however, is based upon studies using short periods of shear stress, which may be perceived by cells as an inflammatory insult that resolves with time. Previous work from our group has shown that chronic exposure to shear stress (up to 7 d) is not associated with increases in COX-2 expression in porcine aortic endothelial cells (25). Here we confirm our earlier observations, showing that COX-1, but not COX-2 immunoreactivity was abundant in human aortic

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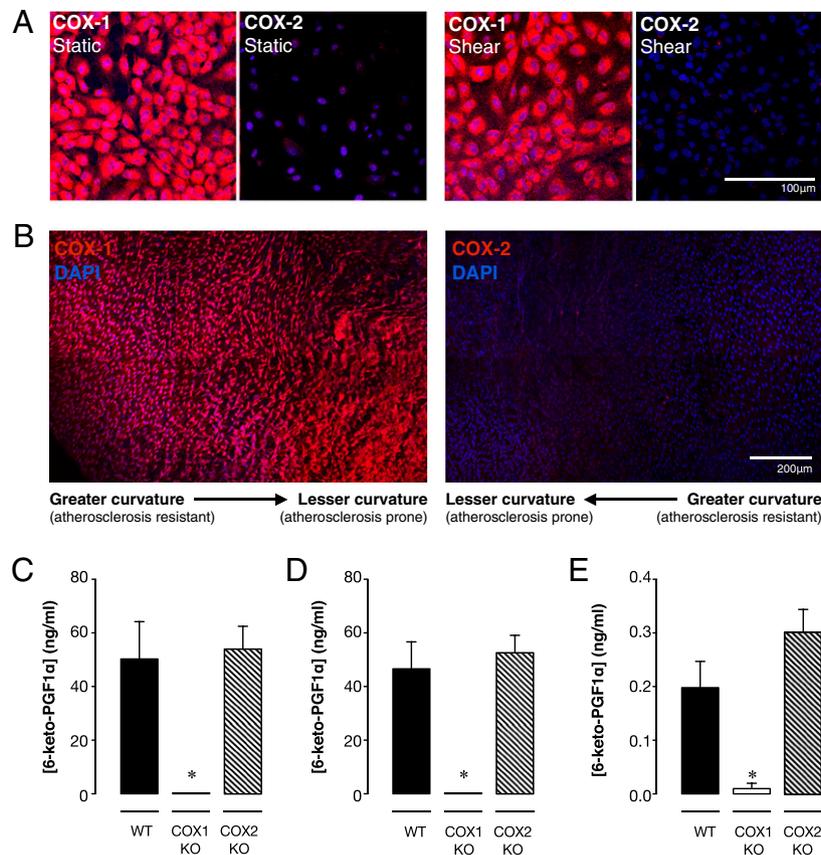


Fig. 1. Expression and activity of COX-1 and COX-2 in endothelium and vessels. (A) Abundant COX-1 but sparse COX-2 immunoreactivity was detected in human aortic endothelial cells cultured under static conditions or after 7 d of shear. Data are representative of six wells using cells from three separate donors. (B) Abundant COX-1, but sparse COX-2 immunoreactivity (red) was detected in the endothelium of the mouse aortic arch. Images are from aortic arches from 12-wk-old wild-type male mice and are representative of data from at least $n = 6$ each of young (10–12 wk) and aged (50–60 wk) male and female mice. COX activity was stimulated in (C) aortic arches or (D) thoracic aorta by placing tissue immediately (<10 min after death) into media containing the calcium ionophore, A23187 (50 μ M) and incubation for 30 min or (E) placing rings of thoracic aorta into DMEM alone, and after a 1-h equilibration period, replacing media and incubating for 30 min. Prostacyclin release was measured as the breakdown product 6-keto PGF $_{1\alpha}$ by enzyme immunoassay. Data are the mean \pm SEM for tissue from $n = 4$ (C–E) mice aged 10–12 wk. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; * $P < 0.05$ vs. wild type.

endothelial cells cultured under static conditions (Fig. 1A). Furthermore, growing cells under shear stress conditions for 7 d did not increase COX-2 immunoreactivity (Fig. 1A). COX-2 expression (fluorescence intensity) could, however, be increased by the addition of lipopolysaccharide (LPS; control: 1.2 ± 0.4 ; plus 0.1 μ g/mL LPS: 6.0 ± 1.8 ; $n = 6$). This result is in accordance with previous observations, which taken together, indicate that whereas acute periods of shear stress induce COX-2 expression, this is a transient response of the cells and does not result in sustained COX-2 expression (20, 23, 24, 26, 27). Studies with isolated endothelial cells can only tell us so much about the situation in blood vessels where cells are exposed to complex patterns of shear stress for the entire life span of the animal. To address the role of COX-2 in whole blood vessels more directly, we imaged COX immunoreactivity in the endothelium of the mouse aortic arch. As an experimental model this offers both well-characterized endothelial phenotypes and a vessel architecture that maps to defined complex shear patterns (28–30). As we found in human endothelial cells, endothelium of the mouse aortic arch from wild-type mice exhibited abundant COX-1 immunoreactivity with sparse levels of COX-2 (Fig. 1B). In line with what we found for isoform expression, prostacyclin release, measured as its breakdown product, 6-keto-PGF $_{1\alpha}$, from aortic arch segments stimulated with calcium ionophore (Fig. 1C), was found to be COX-1 and not COX-2 dependent. Similar results

were obtained in parallel studies using thoracic aorta segments stimulated with calcium ionophore (Fig. 1D) or unstimulated conditions (Fig. 1E), and in which release could be abolished (reduced <95%) by the nonselective COX-1/COX-2 inhibitor, diclofenac (Fig. S1). Similar results were recently published by Lui and colleagues (31). These experiments clearly demonstrate that in the aorta, COX-1 and not COX-2 mediates prostacyclin release. The finding that COX-1 immunoreactivity is expressed in blood vessels is not new; others have shown this in a variety of tissues and using a variety of imaging techniques (15–19). It has been suggested, however, that COX-2 is a very unstable protein, which could be rapidly lost in postmortem processing of tissues explaining its absence in immunohistochemical studies (1). Indeed, here we report that human COX-2 protein induced by IL-1 β in the human lung epithelial cell line, A549 (32), was relatively unstable with a half-life of 2–4 h (Fig. S2). In our studies, aortic arch tissue was fixed as rapidly as possible (<5 min postmortem) and prostacyclin release assays performed ≤ 10 min postmortem, well within the half-life for COX-2 protein (Fig. S2). To test directly the stability of COX-2 in vascular tissue, we removed aorta from wild-type, COX-1 $^{-/-}$, and COX-2 $^{-/-}$ mice and followed the time course of COX expression and prostacyclin release. As before, we saw very little COX-2 expression in the endothelium of the aorta up until 2 h postmortem (Fig. 2A and B). Consequently, up to this time point, prostacyclin release

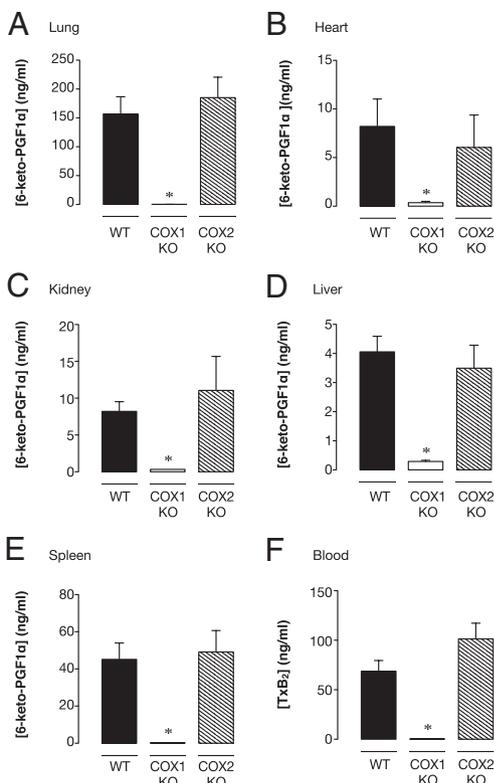


Fig. 4. COX activity in a range of mouse organs measured immediately after death. Lung (A), heart (B), kidney (C) liver (D), spleen (E), and blood (F) were taken from mice and immediately placed into DMEM and stimulated with A23187 (50 μ M). After 30 min, conditioned media were collected and COX activity was measured by the formation of 6-keto-PGF_{1 α} by solid tissue pieces, or by the formation of TxB2 by blood. Data are the mean \pm SEM for $n = 4$ mice. Data are analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; * $P < 0.05$ vs. wild type.

observations and those of others in the literature, we performed experiments in which prostacyclin levels in the circulation were measured as its direct breakdown product in plasma, 6-keto-PGF_{1 α} . Circulating levels of prostacyclin are low, but can be increased by i.v. administration of bradykinin (36), which selectively activates endothelial cells but not platelets. Basal plasma levels of 6-keto-PGF_{1 α} were relatively low, but detectable (Fig. 6A). Bradykinin (100 nmol/kg, i.v.) increased plasma levels of 6-keto-PGF_{1 α} approximately fivefold (Fig. 6A). Both basal and bradykinin-stimulated 6-keto-PGF_{1 α} levels were greatly depressed in COX-1^{-/-} mice, but unaffected in COX-2^{-/-} mice (Fig. 6A), showing that endothelial-derived prostacyclin in vivo, as in vitro, is driven by the activity of COX-1 and not COX-2. Furthermore, these results indicate that urinary PGI-M is not a good correlate of circulating levels of prostacyclin breakdown product, 6-keto-PGF_{1 α} . In line with this, we found that the COX-2 inhibitor, parecoxib, at concentrations that spared the platelet (Fig. 6B) but inhibited COX-2 activity >85% ex vivo (Fig. 6C) had no effect on basal (Fig. 6D) or bradykinin-stimulated (Fig. 6E) prostacyclin release in the circulation. These data further corroborate the idea that prostacyclin in the circulation is driven by COX-1 and that urinary markers of prostacyclin are not reflective of the situation in the circulation.

In healthy human endothelium and in healthy laboratory animals, therefore, COX-1, and not COX-2, drives prostacyclin, and urinary PGI-M levels are not reflective of the prostacyclin in the circulation. With the idea that COX-2 in endothelium generally drives prostacyclin production in healthy individuals

removed, we must seek new answers to why COX-2 inhibitors increase the risk of cardiovascular events. Our group has previously published data showing that COX-2 inhibitors can have an acetaminophen-like effect, inhibiting COX-1 in low lipid peroxide environments, and this may now warrant further research (15). Furthermore, COX-2 in the kidney has well-defined effects on blood pressure, and so the role of COX-2 and NSAID pharmacology in renal function may need to be revisited and associations between COX-2 and blood pressure regulation more deeply investigated (7, 10). COX-2 inhibition may also regulate cardiovascular health indirectly by functions in nerves or in the gut (8), by influences within the brain where COX-2 is constitutively expressed (37), or by affecting the progression of inflammatory disease within the circulation (9). These and other possibilities now need to be properly investigated in order for us to fully understand the effects of NSAIDs upon cardiovascular health. The prevailing dogma that COX-2 in the endothelium supports normal vascular prostacyclin production is simply not supported by evidence from immunohistochemical, pharmacological, or physiological investigations.

Materials and Methods

Mice. COX-1^{-/-} (38) and COX-2^{-/-} (39) mice, were backcrossed for more than seven generations onto a C57BL/6 background (Harlan). Wild-type mice were generated by intercrossing C57BL/6 backcrossed COX-1^{+/-} and COX-2^{+/-} mice. All mice used in the study were genotyped (40) to establish COX-1 and COX-2 status before use. Unless otherwise stated, experiments were performed on male and female 10- to 12-wk-old mice. All animal procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and after local ethical review (Imperial College London Ethical Review Panel).

Cell Culture. Human aortic endothelial cells were purchased from Lonza and cultured according to manufacturer instructions using recommended media (EGM-2 containing hydrocortisone). As hydrocortisone can inhibit the induction of COX-2, 3 d before shear experiments, cells were switched to hydrocortisone-free EGM-2. Cells were seeded onto Transwell filters coated with fibronectin (50 μ g/mL; Sigma) and allowed to equilibrate for 24 h before further experimentation. To assess the effect of shear stress, a PS-300

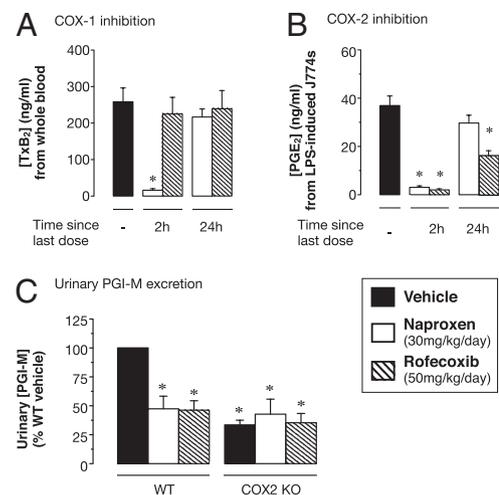


Fig. 5. Effect of naproxen and rofecoxib in vivo on activities of COX-1 and COX-2 and urinary prostacyclin metabolite (PGI-M). Mice were dosed by oral gavage daily for 7 d with naproxen (30 mg/kg), rofecoxib (50 mg/kg), or vehicle and COX inhibitory activity of their blood measured ex vivo (A) in A23187-stimulated whole blood for COX-1 or (B) on LPS-induced murine J774 macrophages for COX-2 ($n = 4$). (C) PGI-M levels in urine collected from $n = 4$ metabolic cages, each containing three to four mice were analyzed, normalized to vehicle control as described previously for studies of this type (33). Data are the mean \pm SEM. Data in A and B were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test, and data in C by one-sample t test; * $P < 0.05$ vs. wild-type vehicle.

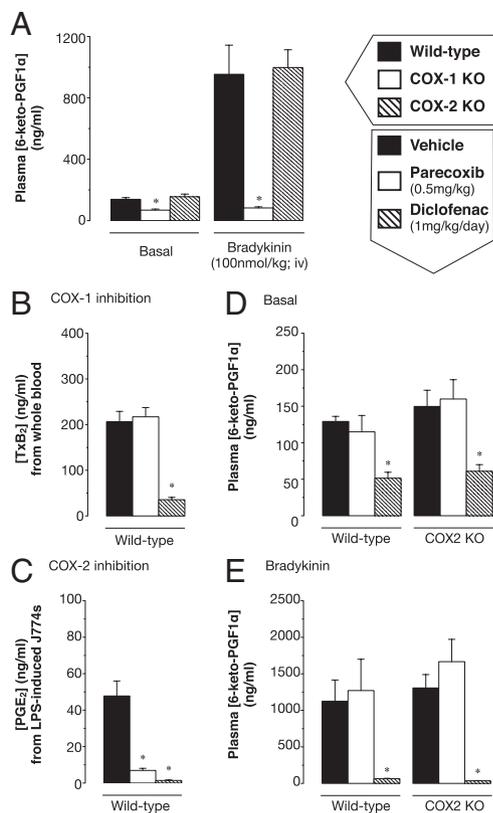


Fig. 6. Role of COX-1 versus COX-2 in driving prostacyclin production in the circulation in vivo. (A) Prostacyclin release in vivo was measured as 6-keto-PGF_{1α} levels in plasma under control (basal) conditions and after administration of bradykinin (100 nmol/kg; i.v.) in wild-type, COX-1^{-/-}, and COX-2^{-/-} mice ($n = 6$). COX inhibitory activity of blood from mice treated with i.v. parecoxib (0.5 mg/kg), diclofenac (1 mg/kg), or vehicle was measured ex vivo (B) in A23187-stimulated whole blood for COX-1 or (C) on LPS-induced murine J774 macrophages for COX-2 ($n = 4$). The effect of the parecoxib and diclofenac on prostacyclin release in vivo in wild-type mice was measured as 6-keto-PGF_{1α} levels in plasma under (D) control (basal) conditions and (E) after administration of bradykinin (100 nmol/kg; i.v.). Data are mean \pm SEM for $n = 6$ male and female, 10- to 12-wk-old mice per genotype. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; * $P < 0.05$ vs. wild type.

orbital shaker (Grant Instruments) was used as previously described (25). In some cultures, LPS (0.1 μ g/mL; from *Escherichia coli* serotype 0111:B4; Sigma) was added to the media as a positive control. Cells were incubated under either static conditions (no shear) or shear stress for 7 d, with media replaced every 2 d. At the end of the experiment, media was removed and cells were fixed in 5% (vol/vol) neutral buffered formalin (Sigma) as described previously (25).

Immunohistochemistry and Confocal Imaging. Mice euthanized with CO₂ were immediately perfused across the heart with PBS (20 mL) followed by 5% formalin (20 mL), and the aortic arch was carefully removed. The aortic tissue was then blocked (20% normal goat serum; Vector Laboratories) and permeabilized (0.1% Triton X-100; Sigma), treated with rabbit antimouse COX-1 (1:50; Cayman Chemical) or rabbit antimouse COX-2 (1:50; Cayman Chemical) primary antibodies, followed by Alexa 594-conjugated goat antirabbit IgG secondary antibodies (1:100; Invitrogen). Tissues were counterstained with Alexa 488-conjugated rat antimouse CD31 (1:100; Biolegend) and DAPI (25 μ g/mL; Invitrogen). After staining, aortic rings were cut open to reveal the luminal surface, mounted flat between a glass slide and coverslip with aqueous hard-set media (Vector Labs), and pressed until the media had firmly set. Formalin-fixed Transwell filters on which human aortic endothelial cells were grown were stained as aortic arches except that cells were blocked in 1% BSA, permeabilized in 0.2% Triton X-100, and rabbit anti-human COX-1 (1:250; Cayman Chemical), rabbit antihuman COX-2 (1:250;

Cayman Chemical). Stained filters were mounted on glass slides under coverslips with aqueous media (Vector Labs) as we previously described (25).

The luminal surface of aortic rings and the human aortic endothelial cell-coated surface of the Transwell filters were visualized with a Leica SP5 inverted confocal microscope using a 40 \times objective oil immersion lens. Laser and gain settings were fixed at the beginning of each imaging protocol. In aortic arch preparations, areas corresponding to the lesser and greater curvature were determined by tissue orientation and confirmed by the cell morphology in the CD31⁺ endothelial cell layer as described previously (28, 29). For both aortic arches and human aortic endothelial cell-coated Transwell filters, nonspecific binding was excluded by subtracting the fluorescence of tissue/cells in which the primary antibody was omitted from the staining protocol. The COX-1 and COX-2 immunoreactivity was quantified as mean fluorescence intensity using Fluorescence Lite software (Leica Microsystems) (25). The specificity of the antibodies used was confirmed by the ability of specific blocking peptides to quench immunoreactivity (Fig. S3).

In Vitro COX Activity Bioassays. Aortic tissue, blood and various organs from animals perfused with PBS, were carefully dissected into small pieces (~2-mm rings for aortic tissue, ~25 mm³ for solid organs) and placed into individual wells of 48- or 96-well microtiter plates containing DMEM (200 mM L-glutamine; Sigma). For studies where tissue was incubated ex vivo for up to 7 d, DMEM was additionally supplemented with FCS (10%; Sigma), penicillin (100 units/mL; Sigma), streptomycin (100 μ g/mL; Sigma), 2.5 μ g/mL amphotericin B (2.5 μ g/mL; Sigma), and nonessential amino acid solution (Sigma). In some studies, tissues were treated with nonselective COX inhibitor diclofenac (100 μ M; Sigma) and/or the calcium ionophore, A23187 (50 μ M; Sigma). These details, as well as the period of incubation of tissues ex vivo are defined in individual figure legends. In some studies, after bioassay, aortic rings were fixed for 10 min in 5% formalin and COX-1 and COX-2 immunoreactivity evaluated as above.

In separate studies, organs were removed and homogenized to assay COX activity in cell press preparations. Prostacyclin was measured in conditioned media or homogenate supernatants, by selective enzyme immunoassay for 6-keto-PGF_{1α} (a stable breakdown product of prostacyclin; Cayman Chemical). For blood COX activity, thromboxane release was measured using a selective ELISA for the breakdown product thromboxane B₂ (Cayman Chemical).

Western Blotting. Snap-frozen tissues were homogenized in PBS containing EDTA (10 mM), Triton-X 100 (1%), polymethylsulfonyl fluoride (1 mM), and Roche protease inhibitor mixture (1 \times) using a Precellys 24 homogenizer. Protein concentration in homogenates was determined by Bradford assay and samples were separated by SDS/PAGE (20 μ g/mL total protein per lane). Protein was transferred onto nitrocellulose membranes, which were then probed with anti-COX-1 (1:1,000; Cayman Chemical), anti-COX-2 (1:1,000; Cayman Chemical), anti- β -actin (1:10,000; Dako), or anti-GAPDH primary antibodies (1:2,000; Abcam). Immunoreactivity was visualized using corresponding HRP-conjugated secondary antibodies (Dako) and enhanced chemiluminescent detection (GE Amersham).

Urinary Prostacyclin Metabolite Excretion. Mice were treated for 7 d with rofecoxib (50 mg/kg; Merck), naproxen (30 mg/kg; Sigma), or vehicle (1% DMSO; VWR) by once-daily oral gavage in a randomized triple crossover fashion with 14 d wash out between treatments. For the final 2 d of each treatment, mice were housed in metabolic cages for the collection of urine, and the urinary levels of PGI-M were determined by gas chromatography-tandem mass spectrometry as previously described (34). To confirm the effectiveness and selectivity of treatments, 2 or 24 h after the final dose of NSAID was administered, blood was collected for measurement of COX-1 and COX-2 inhibitory activity as we have previously described (41). COX-1 inhibition was determined by stimulating whole blood with A23187 (50 μ M, 30 min) and measurement of platelet TxB₂ formation by enzyme immunoassay (Cayman Chemical). COX-2 inhibitory activity was determined by applying plasma to J774 murine macrophages in which COX-2 had been induced with LPS (from *Escherichia coli* serotype 0111:B4; 10 μ g/mL; Sigma), before stimulation with A23187 (50 μ M, 30 min) and measurement of PGE₂ formation by enzyme immunoassay (Cisbio).

Circulating Prostacyclin Measurement in Vivo. Under isoflurane anesthesia, the right jugular vein and left carotid artery of wild-type, COX-1^{-/-} and COX-2^{-/-} mice were cannulated. Where required, the selective COX-2 inhibitor, parecoxib (0.5 mg/kg; Pfizer), the nonselective COX-1/COX-2 inhibitor diclofenac (1 mg/kg; Novartis) or vehicle was administered via the venous cannula. After a 30-min stabilization period, 0.2 mL arterial blood was withdrawn and 0.2 mL warm saline infused into the venous cannula. After a further 5 min of stabilization, bradykinin (100 nmol/kg; Tocris Bioscience) was administered i.v. and 0.5 mL arterial blood collected 5 min later, before the animal was euthanized. Plasma was separated from heparinized (10 units/mL; Leo Laboratories) blood

and the levels of the prostacyclin breakdown product 6-keto-PGF_{1α} measured in pre- and postbradykinin plasma samples by enzyme immunoassay (Cayman Chemical). In parallel studies, 30 min after treatment with parecoxib, diclofenac, or vehicle, blood was collected for measurement of COX-1 and COX-2 inhibitory activity *ex vivo* as described above.

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