Correction

**MEDICAL SCIENCES**


The authors note that Fig. 6 appeared incorrectly. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

![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-ketoPGF$_{1\alpha}$ 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-ketoPGF$_{1\alpha}$ levels in plasma under (D) control (basal) conditions and (E) after administration of bradykinin (100 nmol/kg; i.v.). Data are mean ± 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.](www.pnas.org/cgi/doi/10.1073/pnas.1220901110)
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 prostanooids. 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 prostanooids, 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-PGF₁α (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 metabolites 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).

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endothelial cells cultured under static conditions (Fig. 1A).

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 μM) 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 PGF₁α by enzyme immunoassay. Data are the mean ± 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.
was dependent upon COX-1, and not COX-2 (Fig. 2C). Paradoxically, however, we found that at time points after 2 h, COX-2 immunoreactivity was clearly detected in the endothelium of aorta from wild-type mice. In line with this, COX-2 activity took over from COX-1 as the main isoform driving prostacyclin release in tissue maintained in culture from 4 h up until 7 d (Fig. 2C and D). Clearly, COX-2 is not present in healthy tissues, but can be rapidly induced postmortem.

**Microvascular COX Expression and Activity ex Vivo.** The majority of the endothelium in the body is within organs in which different local stimuli will be present than in the aorta. To address this we investigated the expression of COX in lungs and hearts and found they contained abundant levels of COX-1 with minimal levels of COX-2 protein (Fig. 3A). Importantly, as in isolated vessels, the release of prostacyclin by segments (Fig. 3B and C) or homogenates (Table S1) of lung or heart was completely dependent on COX-1. We also found that prostacyclin release from the endocardium, sampled from medium incubated within the chamber of the left ventricle, was dependent on COX-1 and not COX-2 (Fig. 3D). In a separate study where paired tissue samples from various organs were rapidly removed postmortem (<10 min) and incubated in parallel, we found that COX activity in lung, heart, kidney, liver, spleen, and blood was, in each case, dependent upon COX-1 and not COX-2 (Fig. 4).

**In Vivo Endothelial Cell COX Activity.** The experiments above clearly show that for all of the in vitro assays of COX activity we conducted, COX-1 regulates prostacyclin production. In vitro studies may not, however, reflect what is happening in vivo. The notion that COX-2 regulates prostacyclin production in the circulation comes from measurement of the urinary prostacyclin marker, PGI-M, in the urine of human volunteers (13, 14) and laboratory animals (33). In the current study, we performed similar experiments using wild-type and COX-2−/− mice. Mice were dosed daily for 7 d with either the nonselective COX-1/COX-2 inhibitor naproxen (30 mg kg−1 d−1 orally), or the selective COX-2 inhibitor, rofecoxib (50 mg kg−1 d−1 orally). Ex vivo validation studies, measuring circulating drug activity in the plasma of mice confirmed that naproxen inhibited both COX-1 and COX-2, whereas rofecoxib selectively inhibited COX-2 (Fig. 5A and B). Urine was collected from these animals, PGI-M measured by mass spectrometry (34), and data were analyzed as described previously (33). In line with what others have found in COX-2−/− mice, we found that levels of PGI-M were reduced compared with those found in urine of wild-type mice (Fig. 5C). Moreover, in addition to naproxen, the COX-2 inhibitor, rofecoxib, inhibited urinary PGI-M in wild-type, but not in COX-2−/− mice (Fig. 5C). These observations are in accordance with those of others in the field and clearly point to urinary PGI-M being driven by a COX-2-dependent pathway, yet these findings are at direct odds to what we find in vessels and organs of mice ex vivo. We reasoned that perhaps urinary markers of prostacyclin are not reflective of levels in the circulation. Most recently, others have found the same to be true for urinary markers of thromboxane (TX-M) (35). To consolidate our...
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inhibitor, parecoxib, at concentrations that spared the platelet

6-keto-PGF

6-keto-PGF

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Materials and Methods

Mice. COX1−/− (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 con-
ducted in accordance with the Animals (Scientific Procedures) Act 1986 and

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 in-

duction of COX-2, 3 d before shear experiments, cells were switched to hy-

drocortisone-free EGM-2. Cells were seeded onto Transwell filters coated

with fibronectin (50 μg/mL; Sigma) and allowed to equilibrate for 24 h be-

fore further experimentation. To assess the effect of shear stress, a PS-300

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-

PGF1α. Circulating levels of prostacyclin are low, but can be increased by i.v. administration of bradykinin (36), which selec-
tively activates endothelial cells but not platelets. Basal plasma levels of 6-keto-PGF1α were relatively low, but detectable (Fig.

6A). Bradykinin (100 nmol/kg, i.v.) increased plasma levels of 6-keto-PGF1α approximately fivefold (Fig. 6A). Both basal and

bradykinin-stimulated 6-keto-PGF1α 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. Furthmore, these results indicate that urinary PGI-M is not a good correlate of circulating levels of prostacyclin breakdown product, 6-keto-PGF1α. 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 re-

fective 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 gen-

erally 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 previ-

ously 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 phar-

macology in renal function may need to be revisited and asso-

ciations 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 consti-

tively expressed (37), or by affecting the progression of in-

flammatory 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, pharmaco-

logical, or physiological investigations.

Materials and Methods

Mice. COX1−/− (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 con-
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Escherichia coli

Laser

Objective oil immersion lens.

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Wild-type

CoX-1 Ko

CoX-2 Ko

Vehicle

Parecoxib

(0.5 mg/kg)

Diclofenac

(1 mg/kg)

Plasma [6-keto-PGF1α (ng/mL)]

Basal

Bradykinin (150 mg/kg, i.v.)

Plasma 6-keto-PGF1α from whole blood

COX-1 inhibition

Wild-type

CoX-1 Ko

CoX-2 Ko

Vehicle

Parecoxib

(0.5 mg/kg)

Diclofenac

(1 mg/kg)

Plasma [PGD2 (ng/mL)]

Basal

Bradykinin (150 mg/kg, i.v.)

Plasma PGD2 from whole blood

COX-1 inhibition

Wild-type

CoX-1 Ko

CoX-2 Ko

Vehicle

Parecoxib

(0.5 mg/kg)

Diclofenac

(1 mg/kg)

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; VWIR) by once-daily oral gavage in a randomized triple crossover fashion with 14 d washout periods 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 μM, 30 min) and measurement of platelet thromboxane B2 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 μg/mL) and measurement of measurement of PGE2 formation by enzyme immunoassay (Cisbio). The effect of the nonselective COX inhibitor diclofenac (100 μM; Sigma) and the calcium ionophore, A23187 (50 μM; Sigma), on the levels in plasma under control (basal) conditions and after administration of bradykinin (100 nmol/kg; i.v.); Roche protease inhibitor mixture (1x) using a PreScylla 24 homogenizer. Protein concentration in homogenates was determined by Bradford assay and samples were separated by SDS-PAGE (20 μg/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).

In vitro COX Activity Bioassays. Aortic tissue, blood and various organs from animals perfused with PBS were dissected into small pieces (2-mm rings for aortic tissue, ~25 mm² for solid organs) and placed into individual wells of 48- or 96-well microtiter plates containing DMEM (200 mM l-glutamie; 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 μg/mL; Sigma), 2.5 μg/mL amphotericin B (Sigma), and nonessential amino acid solution (Sigma). In some studies, tissues were treated with nonselective COX inhibitor diclofenac (100 μM; Sigma) and/or the calcium ionophore, A23187 (50 μM; 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-PGF1α (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%), polyethylene sulfonate fluoride (1 mM), and Roche protease inhibitor mixture (1x) using a Precellys 24 homogenizer. Protein concentration in homogenates was determined by Bradford assay and samples were separated by SDS-PAGE (20 μg/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).
and the levels of the prostacyclin breakdown product 6-keto-PGF₁α measured in pre- and postbradykinin plasma samples by enzyme immunoassay (Cayman Chemical). In parallel studies, the effect of treatment with parecoxib, diclofenac, or vehicle, blood was collected for measurement of COX-1 and COX-2 inhibitory activity ex vivo as described above.