Cyclo-oxygenase-1 and not cyclo-oxygenase-2 is responsible for physiological production of prostacyclin in the cardiovascular system

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Abstract

Prostacyclin is an anti-thrombotic hormone produced by the endothelium, whose production is dependent on cyclo-oxygenase (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 shows 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 in order to move forward with drug discovery and to enable more informed prescribing advice.

Introduction

Prostacyclin is an anti-thrombotic 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 cyclo-oxygenase (COX) enzymes, which convert arachidonic acid into prostaglandin H_2, 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 non-steroid anti-inflammatory drugs (NSAIDs; e.g. ibuprofen, diclofenac) as well as COX-2-selective drugs (e.g. celecoxib, 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 associated with the use of 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-$PGF_{1\alpha}$ (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 shows 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 levels 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 in order 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 employing 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 days) 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 endothelial cells cultured under static conditions (Figure 1a). Furthermore, growing cells under shear stress conditions for 7 days did not increase COX-2 immunoreactivity. COX-2 expression (fluorescence intensity) could however, be increased by the addition of lipopolysaccharide (LPS) (control; 1.2±0.4: plus 0.1μg/ml LPS; 6.0±1.8; n=6). This result is in accordance with previous observations which taken together indicate that whilst 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. In order 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 exhibited abundant COX-1 immunoreactivity with sparse levels of COX-2 (Figure 1b). In line with what we found for isoform expression, prostacyclin release, measured as 6-keto$PGF_{1\alpha}$, from aortic arches stimulated with calcium ionophore (Figure 1c) was found to be COX-1 and not COX-2 dependent. Similar results were obtained in parallel studies using thoracic aorta stimulated with calcium ionophore (Figure 1d) or un-stimulated conditions (Figure 1e) and, in both cases, release could be abolished (reduced <95%) by the non-selective COX-1/COX-2 inhibitor, diclofenac (Supplementary Figure 1). 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 (see (15-19)). It has been suggested, however, that COX-2 is a very unstable protein, which could be rapidly lost in *post mortem* processing of tissues explaining its absence in immunohistochemical studies (1). Indeed, here we report that human COX-2 protein induced by IL-1β in the human lung epithelial cell line, A549(32), was relatively unstable with a half-life of 2-4 hours (Supplementary Figure 1). In our studies, aortic arch tissue was fixed as rapidly as possible (<5 min *post mortem*) and prostacyclin release assays performed ≤10 min *post mortem*, well within the half-life for COX-2 protein (Supplementary Figure 2). In order 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 hours *post mortem* (Figure 2a-c). Consequently, up to this time point, prostacyclin release was dependent upon COX-1, and not COX-2 (Figure 2d). Paradoxically, however, we found that at time points after 2 hours, 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 hours up until 7 days (Figure 2d,e). Clearly, COX-2 is not present in healthy tissues, but can be rapidly induced *post mortem*.

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. In order 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 (Figure 3a). Importantly, as in isolated vessels, the release of prostacyclin by segments (Figure 3b-c) or homogenates (Table 1) 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 (Figure 3d). In a separate study where paired tissue samples from various organs were rapidly removed *post mortem* (<10 minutes) 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 (Figure 4).

In vivo endothelial cell COX activity

The experiments above clearly show that for all 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 with either the non-selective COX-1/COX-2 inhibitor naproxen (30mg/kg/day), or rofecoxib (50mg/kg/day), which is a selective COX-2 inhibitor. *Ex vivo* validation studies, measuring circulating drug activity in the plasma of mice confirmed that naproxen inhibited both COX-1 and

COX-2, whilst rofecoxib selectively inhibited COX-2 (Figure 5a,b). Urine was collected from these animals, PGI-M measured by mass spectrometry (34) and data analyzed as described previously (33). In line with what others have found in COX-2 deficient mice, we found that levels of PGI-M were reduced compared to those found in urine of wild-type mice (Figure 5c). Moreover, in addition to naproxen, the COX-2 inhibitor, rofecoxib, inhibited urinary PGI-M in wild-type, but not in COX-$2^{-/-}$ mice (Figure 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). In order to consolidate our 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$PGF_{1\alpha}$. Circulating levels of prostacyclin are low, but can be increased by intravenous administration of bradykinin (36), which selectively activates endothelial cells but not platelets. Basal plasma levels of 6-keto$PGF_{1\alpha}$ were relatively low, but detectable (Figure 6). Bradykinin (100nmol/kg, i.v.) increased plasma levels of 6-keto-$PGF_{1\alpha}$ approximately 5-fold (Figure 6a). Both basal and bradykinin stimulated 6-keto-$PGF_{1\alpha}$ levels were greatly depressed in COX-$1^{-/-}$ mice, but unaffected in COX-$2^{-/-}$ mice (Figure 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 metabolite, 6-keto-$PGF_{1\alpha}$. In line with this, we found that the COX-2 inhibitor, paracoxib, at concentrations that spared the platelet (Figure 6b) but inhibited COX-2

activity >85% ex vivo (Figure 6c) had no effect on basal (Figure 6d) or bradykinin-stimulated (Figure 6e) prostacyclin release in the circulation. This data further corroborates 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 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 re-visited 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), 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 vascular prostacyclin production is simply not supported by evidence from immunohistochemical, pharmacological, or physiological investigations.

Materials and Methods

Mice

COX1$^{-/-}$ (37) and COX-2$^{-/-}$ (38) mice, were back-crossed for >7 generations onto a C57Bl/6 background (Harlan, UK). Wild-type mice were generated by inter-crossing C57Bl/6 back-crossed 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 10-12 week old mice. All animal procedures were conducted in accordance with Animals (Scientific Procedures) Act 1986 and after local ethical review.

Cell culture

Human aortic endothelial cells were purchased from Lonza and cultured according to manufacturers instructions using recommended media (EGM-2 containing hydrocortisone). As hydrocortisone can inhibit the induction of COX-2, 3 days prior to shear experiments cells were switched to hydrocortisone-free EGM-2. Cells were seeded onto Transwell filters coated with fibronectin (50μg/ml; Sigma) and allowed to equilibrate for 24 hours before further experimentation. To assess the effect of shear stress, a PS-300 orbital shaker (Grant Instruments) was used as previously described (25). In some cultures, lipopolysaccharide (LPS; 0.1μg/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 days, with media replaced every 2 days. At the end of the experiment, media was removed and cells were fixed in 2% formalin (Sigma) as described previously (25).

Immunohistochemistry and confocal imaging

Mice euthanised with CO_2 were immediately perfused across the heart with PBS (20ml) followed by 2% formalin (20ml) and the aortic arch was carefully removed. The aortic tissue was then blocked (20% normal goat serum, Vector labs) and permeabilized (0.1% Triton X-100, Sigma), treated with rabbit anti-mouse COX-1 (1:50; Cayman Chemical) or rabbit anti-mouse COX-2 (1:50; Cayman Chemical) primary antibodies, followed by Alexa594-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen). Tissues were counterstained with Alexa488-conjugated rat anti-mouse CD31 (1:100; Biolegend) and DAPI (25ug/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 anti-human COX-2 (1:250; Cayman Chemical). Stained filters were mounted under on glass slides under coverslips with aquous media (Vector Labs) as we previously described (25).

The luminal surface of aortic rings, and the human aortic endothelial cells-coated surface of the transwell filters were visualized with a Leica SP5 inverted confocal microscope using a 40X 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

cells coated transwell filters, non-specific 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 (Supplementary Figure 3).

In vitro COX activity bioassays

Aortic tissue, blood and various organs from animals perfused with PBS, were carefully dissected into small pieces (~2mm rings for aortic tissue, ~25mm^3 for solid organs) and placed into individual wells of 48 or 96 well microtitre plates containing DMEM (200mM L-Glutamine; Sigma). For studies where tissue were incubated *ex vivo* for up to 7 days, DMEM was additionally supplemented with FCS (10%; Sigma), penicillin (100 U/mL; Sigma), streptomycin (100 µg/mL; Sigma), 2.5 µg/mL amphotericin B (2.5 µg/mL; Sigma) and non-essential amino acid solution (Sigma). In some studies tissues were treated with non-selective COX inhibitor diclofenac (100µM; Sigma) and/or the calcium ionophore, A23187 (50µM; 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 minutes in 2% formalin and COX-1 and COX-2 immunoreactivity evaluated as above.

In separate studies organs were removed and homogenized in order to assay COX activity in cell press preparations. Prostacyclin was measured in conditioned media or homogenate supernatants, by selective enzyme immunoassay for 6-keto-$PGF_{1\alpha}$ (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 B_2 (Cayman chemical, USA).

Western blotting

Snap-frozen tissues were homogenised in PBS containing EDTA (10mM), Triton-X 100 (1%), polymethylsulfonyl fluoride (1mM) and Roche protease inhibitor cocktail (1X) using a Precellys 24 homogeniser. Protein concentration in homogenates was determined by Bradford assay and samples separated by SDS-PAGE (20ug/ml total protein/lane). Protein was transferred onto nitrocellulose membranes, which were then probed with anti-COX-1 (1:1000; Cayman Chemical), anti-COX-2 (1:1000; Cayman Chemical), anti-beta-actin (1:10000; Dako) or anti-GAPDH primary antibodies (1:2000; Abcam). Immunoreactivity was visualised using corresponding HRP-conjugated secondary antibodies (Dako) and electrochemiluminescent detection (GE Amersham).

Urinary prostacyclin metabolite excretion

Mice were treated for 7 days with rofecoxib (50mg/kg; Merck), naproxen (30mg/kg; Sigma) or vehicle (1% DMSO; VWR) by once-daily oral gavage in a randomised triple crossover fashion with 14 days wash out between treatments. For the final 2 days of each treatment, mice were housed in metabolic cages for the collection of urine, and the urinary levels of PGI-M (2,3-dinor-6-keto-$PGF_{1\alpha}$) determined by gas chromatography-tandem mass spectrometry as previously described (34). To confirm the effectiveness and selectivity of treatments, 2 or 24 hours 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 (39). COX-1 inhibition was

determined by stimulating whole blood with A23187 (50uM, 30 mins) and measurement of platelet TxB_2 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; 10ug/ml; Sigma)-induced, before stimulation with A23187 (50uM, 30 mins) and measurement of PGE_2 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, paracoxib (0.5mg/kg; Pfizer, UK), the non-selective COX-1/COX-2 inhibitor diclofenac (1mg/kg; Novartis, UK) or vehicle were administered via the venous cannula. After a 30 min stabilisation period, 0.2ml arterial blood was withdrawn and 0.2ml warm saline infused into the venous canula. After a further 5 minutes of stabilisation, bradykinin (100nmol/kg; Tocris Bioscience) was administered intravenously and 0.5ml arterial blood collected 5 mins later before the animal was euthanised. Plasma was separated from heparinized (10U/ml) blood and the levels of the prostacyclin breakdown product 6-keto-$PGF_{1\alpha}$ measured in pre- and post-bradykinin plasma samples by enzyme immunoassay (Cayman Chemical). In parallel studies, 30 mins after treatment with paracoxib, 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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Figure 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 days of shear. Data is representative of 6 wells using cells from 3 separate donors. (b) Abundant COX-1, but sparse COX-2 immunoreactivity (red) were detected in the endothelium of the mouse aortic arch. (a-b) Images are from aortic arches from 12 week old male mice and are representative of data from at least n=6 each of young (10-12 weeks) and aged (50-60 weeks), male and female mice. COX activity was stimulated in aortic arches (c) or thoracic aorta (d) by placing tissue immediately (<10 minutes after death) into media containing the calcium ionophore, A23187 (50μM) and incubation for 30 minutes or (e) placing rings of thoracic aorta into DMEM alone, and after a 1hr equilibration period, replacing media and incubating for 30 mins. Prostacyclin release was measured as the breakdown product 6-keto $PGF_{1\alpha}$ by enzyme immunoassay. Data is the mean ± S.E.M for tissue from n=4 (d-f) mice aged 10-12 weeks. Data was analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; *$p<0.05$ vs. wild-type.

Figure 2 Effect of time post mortem on COX-1 and COX-2 expression and activity in mouse aorta. Representative images of COX-2 (a; red) and COX-1 (b; red) immunoreactivity in the endothelium of aorta from a wild-type mouse incubated *ex vivo* for between 0.5 and 12 hours post-mortem. Pooled mean fluorescence values for (b) COX-2 and (c) COX-1 immunoreactivity from n=4 separate animals, 10-12 week old male and female wild-type mice. (d) COX activity (as 6-keto $PGF_{1\alpha}$) measured in the same aortas over this time course, and (e) COX activity measured 7 days from n=5 separate animals.

Figure 3 COX-1 and COX-2 protein expression and activity in mouse lung and heart. Western blot analysis (a) showed abundant COX-1 with little COX-2 in lungs or hearts wild-type mice. Mouse platelets, which contain only COX-1 and murine J774 macrophages treated with LPS to induce COX-2 were used as controls. COX activity, measured as 6-ketoPGF$_{1\alpha}$ release from intact pieces of (b) lung or (c) heart was measured after 90 minutes equilibration followed by 30 minutes treatment with A23187 (50μM). COX activity within the chamber of the left ventricle (interior) was measured by cannulating the left ventricle of isolated hearts and after equilibration (90 mins) filling with A23187 (50μM) and incubating 30 minutes before collection of the contents. Data is the mean ± S.E.M. for n=6 male and female, 10-12 week old mice. Data is analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; *$p<0.05$ vs. wild-type.

Figure 4 COX activity in a range of mouse organs measured immediately after death. Aorta (a), heart (b), kidney (c) liver (d), blood (e) and spleen (f) were taken from mice and immediately placed into DMEM and stimulated with A23187 (50μM). After 30 minutes, conditioned media was collected and COX activity was measured by the formation of 6-ketoPGF$_{1\alpha}$. Data is the mean ± S.E.M. for n=4 mice. Data is analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; *$p<0.05$ vs. wild-type.

Figure 5 Effect of naproxen and rofecoxib *in vivo* on urinary prostacyclin metabolite (PGI-M). Mice were dosed by oral gavage daily with naproxen (30mg/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 3-4 mice were analyzed normalized to vehicle control as described previously for studies of this type (33). Data is the mean ± S.E.M. Data in (a) and (b) was analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; *p<0.05 vs. wild-type vehicle.

Figure 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 (100nmol/kg; i.v.) in wild-type, COX-1$^{-/-}$ and COX-2$^{-/-}$ mice (n=6). The COX inhibitory activity of blood from mice treated with intravenous paracoxib (0.5mg/kg), diclofenac (1mg/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 these drugs on prostacyclin release *in vivo* was measured as 6-ketoPGF$_{1\alpha}$ levels in plasma under (d) control (basal) conditions and (e) after administration of bradykinin (100nmol/kg; i.v.) Data is mean ± S.E.M. for n=6 male and female, 10-12 week old mice per genotype. Data was analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test; *p<0.05 vs. wild-type.

Tables

6-keto-PGF1a (ng/ml)	**Wild-type**	**COX-1$^{-/-}$**	**COX-2$^{-/-}$**
Heart	27.2 ± 8.6	4.1 ± 1.5 *	29.8 ± 10.9
Lung	661.8 ± 59.7	7.6 ± 2.5 *	894.9 ± 72.4
Kidney	80 ± 13.4	5.5 ± 1.7 *	77.1 ± 15.2
Brain	31.1 ± 2.1	6.2 ± 1.1 *	23.9 ± 2.7

Table 1 Prostacyclin associated COX activity in tissue homogenates of heart, lung, kidney and brain. Organs were snap-frozen immediately *post-mortem*, then homogenized in 50mM Tris buffer. Homogenates were incubated for 20 minutes at 37°C before addition of diclofenac (1 mM) to halt residual COX activity. Homogenates were centrifuged and 6-ketoPGF$_{1\alpha}$ measured in the supernatant by enzyme immunoassay. Data is mean± S.E.M. from n=4 animals. Data was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test; *$p<0.05$ vs. wild-type.