Selective inhibition of prostacyclin synthase activity by rofecoxib

Cristiana Griffoni [a, †], Enzo Spisni [a, †, *], Antonio Strillacci [a], Mattia Toni [a], Markus Michael Bachschmid [b], Vittorio Tomasi [a]

[a] Department of Experimental Biology, University of Bologna, Bologna, Italy
[b] Evans Department of Medicine, Boston University Medical Center, Boston, Massachusetts, USA

Abstract

The development of cyclooxygenase-2 (COX-2) selective inhibitors prompted studies aimed at treating chronic inflammatory diseases and cancer by using this new generation of drugs. Yet, several recent reports pointed out that long-term treatment of patients with COX-2 selective inhibitors (especially rofecoxib) caused severe cardiovascular complicances. The aim of this study was to ascertain whether, in addition to inhibiting COX-2, rofecoxib may also affect prostacyclin (PGI2) level by inhibiting PGI2 forming enzyme (prostacyclin synthase, PGIS). In order to evaluate if selective (celecoxib, rofecoxib) and non-selective (aspirin, naproxen) anti-inflammatory compounds could decrease PGI2 production in endothelial cells by inhibiting PGIS, we analyzed the effect of anti-inflammatory compounds on the enzyme activity by ELISA assay after addition of exogenous substrate, on PGIS protein levels by Western blotting and on its subcellular distribution by confocal microscopy. We also analyzed the effect of rofecoxib on PGIS activity in bovine aortic microsomal fractions enriched in PGIS. This study demonstrates an inhibitory effect of rofecoxib on PGIS activity in human umbilical vein endothelial (HUVE) cells and in PGIS-enriched bovine aortic microsomal fractions, which is not observed by using other anti-inflammatory compounds. The inhibitory effect of rofecoxib is associated neither to a decrease of PGIS protein levels nor to an impairment of the enzyme intracellular localization. The results of this study may explain the absence of a clear relationship between COX-2 selectivity and cardiovascular side effects. Moreover, in the light of these results we propose that novel selective COX-2 inhibitors should be tested on PGI2 synthase activity inhibition.

Keywords: rofecoxib • prostacyclin synthase • nonsteroidal anti-inflammatory agents • cyclooxygenase-2 inhibitors • endothelium

Introduction

The second isoform of cyclooxygenase enzyme discovered (COX-2) is very similar to COX-1 as far as active site structure, catalytic mechanism, products and kinetics are concerned. Yet, two structural differences between the two isoenzymes have important pharmacological and biological consequences. The first difference is that COX-2 active site is larger and more accommodating than that of COX-1 and includes a side pocket not present in COX-1, providing the basis for the development of selective COX-2 inhibitors. Second, only COX-1 (but not COX-2) is negatively regulated in an allosteric manner by

---

arachidonate at low concentrations. Such a difference may permit COX-2 to compete more effectively for the substrate when both the isoenzymes are expressed in the same cells and allow COX-2 to operate independently of COX-1 [1]. When COX-2 is selectively inhibited not only COX-1 becomes more active but it can be upregulated to replace specific functions of COX-2 *in vivo* [2]. While COX-1 is generally constitutively expressed and produces prostanoids playing a homeostatic role, COX-2 is generally absent in cells normally expressing COX-1 under physiological conditions, the only important exception being the kidney macula densa cells [3]. However, COX-2 gene is rapidly and potently turned on following several stimuli and factors and its metabolites are important in inflammation, wound healing, immune response regulation, and angiogenesis [1]. Overexpression of COX-2 occurs at an early stage in the development of human colon cancer as well as in other epithelial malignancies, and it seems to be relevant in promoting tumorigenesis [4, 5].

Prostacyclin (PGI2), the main product of arachidonate metabolism in vascular tissues, is the most potent endogenous inhibitor of platelet aggregation and produces vasodilatation of all vascular beds studied [6–10]. PGI2 may act as an autocrine and paracrine effector to regulate the functions of different cell types by activating specific G-coupled receptors [11] or nuclear receptors belonging to the peroxisomal proliferator-activated receptors (PPARs) family [12]. Prostacyclin shows other new important biological activities. It is thought to be involved in vascular remodeling diseases, such as primary pulmonary hypertension and cardiovascular diseases [13, 14]. It has also been implicated as mediator of cyclooxygenase-2 (COX-2) effects promoting mouse embryo implantation [15]. Finally, PGI2 has been shown to play important roles in the regulation of angiogenesis and apoptosis [16–20].

These observations have stimulated work aimed at elucidating the PGI2 forming enzyme (PGI2 synthase, PGIS) structure and subcellular localization [21].

PGI2 synthase, a microsomal enzyme constitutively expressed in vascular endothelial cells [22], is a 52-kD hemoprotein belonging to the cytocrome P450 (CYP) family. Like other CYP enzymes, PGIS anchors to the membrane with a single N-terminal transmembrane domain, while the bulk of the enzyme is exposed in the cytosolic side of the membranes. The catalytic centre, heme environment, and substrate channel are only partially characterized, because of the lack of an X-ray crystallografic structure [21]. PGI2 synthase promoter is TATA less and GC-rich, consistent with structural features of housekeeping gene. Moreover, it presents a variable number of Sp1 binding sites and its expression varies among individuals [14].

It is now clear that the coupling COX-2/PGI2 synthase is the major event leading to a sustained production of prostacyclin released both in the circulation and into the subendothelium, particularly in activated endothelial cells. The coupling PGIS/COX-2 occurs at the nuclear envelope and endoplasmic reticulum, but also inside membrane microdomains named caveolae, where both PGIS and COX-2 have been detected [18, 23]. Thus, PGIS produces PGI2 as soon as COX-2 is induced and inserted into caveolae, nuclear envelope or endoplasmic reticulum, provided that arachidonate is made available by cytosolic phospholipase A2. COX-2 and PGI2 synthase may be functionally interconnected and both participate to signal transduction events connected to the regulation of processes like angiogenesis and apoptosis [18, 20]. An interesting indirect evidence about the functional association of COX-2 with caveolae is presented by a recent paper showing that nitric oxide synthase – a well-known caveolar enzyme in endothelial cells – is capable of binding, s-nitrosylating and activating COX-2 [24].

It is not clear whether a connection COX-1/PGI2 synthase may exist. COX-1 has been considered a constitutively expressed enzyme having housekeeping functions while COX-2 has been considered an inducible enzyme. However, more recently it appeared that COX-1 can be upregulated in endothelial cells by estrogen [25] in conditions in which also COX-2 appears to be estrogen dependent [26].

A breakthrough in the treatment of inflammatory diseases has been the commercial availability of selective COX-2 inhibitors (coxibs). These compounds, in particular rofecoxib and celecoxib, behave as potent and specific inhibitors of COX-2 and have had a wide diffusion in the treatment of chronic inflammatory diseases [27]. This fact represents an important advance for patients in which aspirin and traditional anti-inflammatory drugs resistance has developed [28], or in the presence of serious gastrointestinal side effects of traditional NSAIDs [29]. Despite the safer gastrointestinal profile of coxibs, unexpectedly serious cardiovascular side effects of coxibs have been registered in long-term treated patients [30–32].

Materials and methods

Nonsteroidal anti-inflammatory drugs (NSAIDs) and Coxibs

The anti-inflammatory drugs used in this study were Acetylsalicylic acid (pharmaceutical preparation), Naproxen sodium salt (Sigma, USA), Celebrex[TM] (Pfizer, USA), and Vioxx[TM] (Merck & Co, USA). Celecoxib and rofecoxib were extracted from Celebrex 200 mg tablets and Vioxx 25 mg tablets, respectively, following a protocol previously described [33]. Extraction efficiency was around 100%. The purity of the compounds was determined by ^1H-^{13}C NMR (nuclear magnetic resonance) spectroscopy. Stock solutions of rofecoxib and celecoxib were prepared in ethyl acetate and kept refrigerated (at 4°C) in a glass tube protected from the light in order to reduce their degradation and photocyclization. For the preparation of stock solutions acetylsalicylic acid and naproxen were dissolved in ethanol or bidistilled water respectively. For cell treatments, diluted solutions of each drug were prepared in dimethylsulfoxide (DMSO).

Cell culture and treatments

Human umbilical vein endothelial (HUVE) cells were isolated from freshly collected umbilical cords and cultured as previously described [22]. Cells were used for experiments between 3rd and 5th passage. Fetal calf serum (FCS) was purchased from Cambrex Biowittaker (USA), the other cell culture reagents were purchased from Sigma (USA).

In order to evaluate the inhibition of prostacyclin production due to the block of COX-2, HUVEC (8×10^4) were seeded in 12-well plates and allowed to grow in complete culture medium, containing 20% FCS. Cells were subsequently treated with different concentrations of the anti-inflammatory drugs, ranging from 10^{-3} M to 10^{-10} M. After 1 hr of NSAID addition, 40 nM phorbol 12-myristate 13-acetate (TPA, Sigma, USA), a well-known stimulator of COX-2 expression was added to cell media. After 24 hrs, cellular supernatants were collected and 6-keto-PGF1α, the stable metabolite of PGI2, was measured by ELISA assay. With the aim of evaluating the enzymatic activity of PGIS after treatment with anti-inflammatory drugs, HUVEC (8×10^4) were seeded in 12-well plates and allowed to grow in complete culture medium. Cells were then treated with different concentrations of the anti-inflammatory drugs, added to complete medium. After 24 hrs, the cell culture medium was replaced with serum-free M199 1X medium, and soon after the exogenous substrate for PGIS, prostaglandin H2 (PGH2, Cayman Chemical, USA), was added at 1 μM concentration for 10 min at room tempera-ture. Supernatants were then collected and 6-keto-PGF1α was measured by ELISA assay. For experiments concerning the determination of kinetics parameters of PGIS enzymatic reaction in the absence or in the presence of rofecoxib, HUVEC were untreated or treated with rofecoxib at 10^{-5} M concentration. After 24 hrs, the cell complete culture medium was replaced with serum-free M199 1X medium and the exogenous substrate PGH2 was added at different concentrations (ranging from 0.25 μM to 5 μM) for 10 min at room temperature. Supernatants were then collected and 6-keto-PGF1α was measured by ELISA assay. ELISA assay kit was purchased from Assay Designs (USA) and used following manufacturer's instructions. All the experiments were performed in triplicate at least three times.

Western Blot and real-time PCR

To analyze PGIS expression levels, HUVE cells, seeded in 25 cm^2 flasks and treated for 24 hrs with anti-inflammatory drugs at different concentrations, were scraped and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitors mixture). Cell lysates were incubated 1 hr on ice and centrifuged at 12,000 g to collect supernatants. Protein concentration in supernatants was evaluated by using the Lowry method. After addition of SDS-PAGE sample buffer and boiling, 40 μg of denatured proteins were separated in 12% SDS-PAGE and then transferred to nitrocellulose papers. After the blotting, nitrocellulose papers were incubated with PGI2 synthase polyclonal antibody (Cayman Chemical, USA) and anti-rabbit IgG HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA). Immunolabeling was visualized by using the ECL procedure (Amersham Biosciences, USA). Bands were quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). Normalization was made against β-actin expression.

For real-time PCR, total RNA was purified by using Eurozol reagent (CELBIO, Milan, Italy) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometry and analyzed by electrophoresis on 1% agarose/formaldehyde denaturing gel to exclude the presence of RNA degradation. Extracted total RNA samples were then treated with DNase I, to remove any genomic DNA contamination, by using DNA-free kit (Ambion, USA). mRNA levels were analyzed by real-time PCR by using a Bio-Rad iCycler system (Bio-Rad, USA) according to the manufacturer's instructions. The specific primer pairs for COX-2 and PGIS were designed by using Beacon Designer 2.0 software. COX-2 primers had the following sequences: 5cctgtgcctgatgattgc3 (forward) and 5ctgatgcgt-gaagtgctg3 (reverse). PGIS primers had the following sequences: 5cggctacctgactctttacg3 (forward) and 5ggc-

gactttgacactgc3 (reverse). The primer pair for the house-keeping β-glucuronidase (GUSB) gene had the following sequences: 5tggtataagaagtatcagaagcc3 (forward) and 5gtatctctctcgcaaaaggaac3 (reverse). Cellular total RNA was reverse-transcribed into cDNAs and then amplified by using a SYBR supermix kit (Bio-Rad, USA) for 40 cycles at 95°C for 30 sec, 53°C for 20 sec, and 72°C for 30 sec. The melting curve data were collected to check PCR specificity. Each cDNA sample was analyzed as triplicate and corresponding samples with no cDNAs were included as negative controls. COX-2 and PGIS mRNA levels for each sample were normalized against GUSB mRNA levels and relative expressions were calculated by using Ct values.

Immunofluorescence and Confocal Microscopy Analysis

To visualize the colocalization between Cav-1 and PGIS, HUVE cells, on sterilized glass coverslips, were washed with PBS, fixed with 4% paraformaldehyde, permeabilised with PBS-Triton X-100 0.05% and then incubated with anti-Cav-1 and anti-PGIS primary antibodies diluted 1:200 in PBS-BSA 10 mg/ml. After washing, cells were incubated with anti-mouse FITC-coniugated and anti-rabbit CY3-coniugated secondary antibodies diluted 1:50 in PBS-BSA 10 mg/ml. Finally, coverslips were mounted in glycerol-PBS medium containing 50 mg/ml DABCO. The imaging was performed on a confocal microscope (Leica, Germany) equipped with an argon/krypton laser. For FITC and CY3 double detection the samples were simultaneously excited with the 488 and 568 nm lines of the argon/krypton laser. Optical sections were obtained at increments of 0.1 μm in the Z-axis and were digitized with a scanning mode format of 512 × 512 pixels and 256 grey levels. The image processing and the volume rendering were performed using the Leica TCS software. Negative controls consisted of samples not incubated with the primary antibody. The double labeling immunofluorescence experiments were carried out avoiding cross reactions between primary and secondary antibodies. In addition, different controls were performed to ensure antibody specificity. The two-dimensional scatter plot diagram of each section was analyzed to evaluate the spatial colocalization of the fluorochromes. For each scatter plot diagram, pixels with highly colocalized fluorochromes, *i.e.* with intensity values greater than 150 grey levels (on a scale from 0 to 255) for both detectors were selected to calculate the colocalization maps and create a binary image.

PGIS activity in bovine aortic microsomal fractions

Bovine aortic microsomal (BAM) fractions, enriched in PGIS, were prepared as previously described [34]. 2 μg

(100 μl) of BAM, diluted in PBS 1X, were pre-incubated with anti-inflammatory drugs at different concentrations for 1 hr at 37°C. Then 50 μl of PGH2 diluted in PBS 1X (final concentration: 1 μM) was added and incubated for 40 sec. The reaction was immediately stopped by addition of 10 μl NaCl/citric acid (2 M). An acidic ether extraction was subsequently performed by adding 600 μl diethyl ether (Merck, Germany) and vortexing for at least 30 sec. at full speed. The upper acidic phase, containing the products of the enzymatic reaction, was removed and placed in a clean test tube. Finally, the solution was evaporated to dryness by vacuum centrifugation in order to remove any trace of organic solvent and the pellet was resuspended in the ELISA buffer. 6-keto-PGF1α was measured by ELISA assay (Assay Designs, USA) following manufacturer's instructions.

Data normalization and statistical analysis

Normalization of 6-keto-PGF1α production was made dividing the 6-keto-PGF1α amount for the number of adherent HUVE cells, evaluated at the end of the experiments by using the acidic phosphatase method [13], and then setting to 100 the values obtained for the controls. Data were expressed as mean ± SEM. Differences were analyzed by one-way ANOVA test, by using SPSS software and considered statistically significant at $P < 0.05$ and $P < 0.01$.

Results

PGIS activity in HUVEC treated with non-selective NSAIDs and selective COX-2 inhibitors

In HUVE cells, TPA strongly increases the expression of COX-2 enzyme, without affecting COX-1 levels, as shown in Figure 1A. The inhibitory doses of non-selective NSAIDs (acetylsalicylic acid and naproxen) and of selective COX-2 inhibitors (celecoxib and rofecoxib) effective on cyclooxygenase activity were determined by measuring the production of 6-keto-PGF1α in HUVE cells stimulated with TPA (Figure 1B). The inhibitory concentration 50% (IC50) of selective COX-2 inhibitors (celecoxib and rofecoxib) were 1.0×10^{-8} M (95% confidence interval, 5.3×10^{-9} - 1.8×10^{-8}) and 5.1×10^{-8} M (95% confidence interval, 3.2×10^{-8} - 7.9×10^{-8}) respectively, while the IC50 of non-selective NSAIDs (acetylsalicylic acid and naproxen) were 8.2×10^{-4} M (95% confidence interval, 5.29×10^{-4} - 1.3×10^{-3}) and 6.3×10^{-4} M (95% confidence interval, $4.5 \times$

10^{-4} - 8.2×10^{-4}) respectively, indicating that NSAIDs affect COX-2 activity in HUVEC even at very low doses.

In order to evaluate a possible non-specific effect of anti-inflammatory agents on PGIS, the most important enzyme downstream cyclooxygenase cascade in endothelial cells, we treated HUVE cells with non-selective NSAIDs (acetylsalicylic acid and naproxen) and selective COX-2 inhibitors (celecoxib and rofecoxib) for 24 hrs and then we supplied exogenous endoperoxide substrate (PGH2) into the medium to by-pass the block of COX activity due to the drugs. We performed several experiments by testing a low dose of each compound, having no effect on COX activity, and two higher doses able to produce the most relevant inhibition of COX-2, as evaluated in Figure 1. As reported in Figure 2, non-selective NSAIDs and celecoxib did not show any significant effect on PGIS activity, both at high doses and at low doses (Fig. 2A-C). Interestingly, rofecoxib showed a significant inhibitory effect on PGIS activity (around 50–60%) at the two doses (10^{-6} M and 10^{-5} M) able to completely block COX-2. Rofecoxib was also found to significantly affect PGIS activity at the low dose (10^{-10} M) ineffective on COX-2 inhibition (Fig. 2D).

Experiments were also performed to evaluate the kinetics parameters of PGIS enzymatic reaction in HUVE cells, in the absence or in the presence of rofecoxib (Fig. 3). The maximum velocity (V_{max}) of the enzyme PGIS in HUVE cells resulted to be around 100 pg/min/10^4 cells and it was decreased to 67 pg/min/10^4 cells when HUVEC were treated with rofecoxib (10^{-5} M concentration) before the addition of the exogenous substrate PGH2. PGIS affinity constant (K_m), calculated around 8 μM, was not altered by the presence of rofecoxib. These data support the hypothesis of rofecoxib as a non-competitive inhibitor of PGIS, determining a reduction of the V_{max} of the enzyme, without affecting its K_m.

PGIS protein levels and intracellular localization in HUVEC treated with non-selective NSAIDs and selective COX-2 inhibitors

In order to verify if non-selective NSAIDs and selective COX-2 inhibitors could alter PGIS expression (at
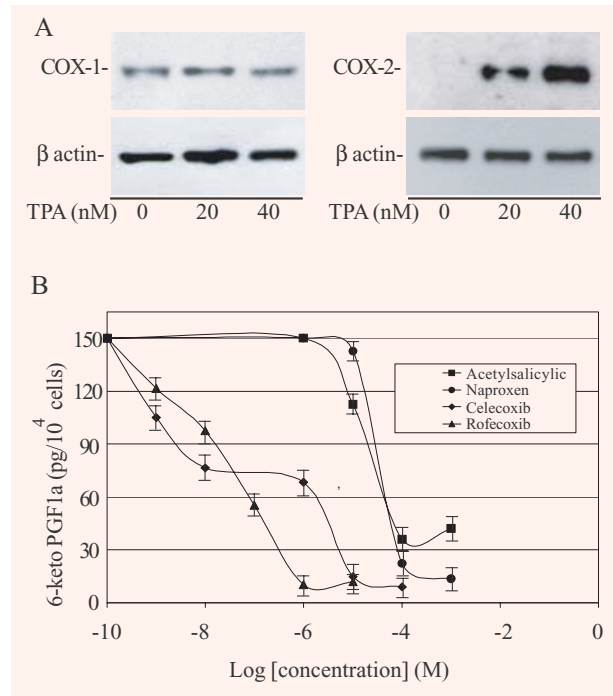

Fig. 1 Effect of non-selective NSAIDs and selective COX-2 inhibitors on cyclooxygenase activity in HUVEC. HUVE cells were stimulated with 20 nM and 40 nM TPA, and analyzed for COX-1 and COX-2 protein levels by Western blot (panel **A**). 40 nM TPA-stimulated HUVE cells (panel **B**) were treated with acetylsalicylic acid, naproxen, celecoxib and rofecoxib at different doses as described under Materials and Methods. The amount of 6-keto-PGF1α released into the cell medium after 24 hrs was evaluated by ELISA assay and it is reported in the graph as pg/10^4 cells ±SEM (n = 9).

transcriptional, translational and post-translational levels), we evaluated by Western blotting PGIS levels in HUVEC treated for 24 hrs with acetylsalicylic acid, naproxen, celecoxib and rofecoxib at the different doses previously tested by ELISA assay. As shown in Figure 4A and B, we observed no significant differences in PGIS levels following the treatments with non-selective NSAIDs and selective COX-2 inhibitors, indicating that the inhibitory effect of rofecoxib on PGIS activity was not associated to an alteration of PGIS expression. Real-time PCR (Fig. 4C) confirmed that no differences in PGIS mRNA expression were induced by rofecoxib treatment.

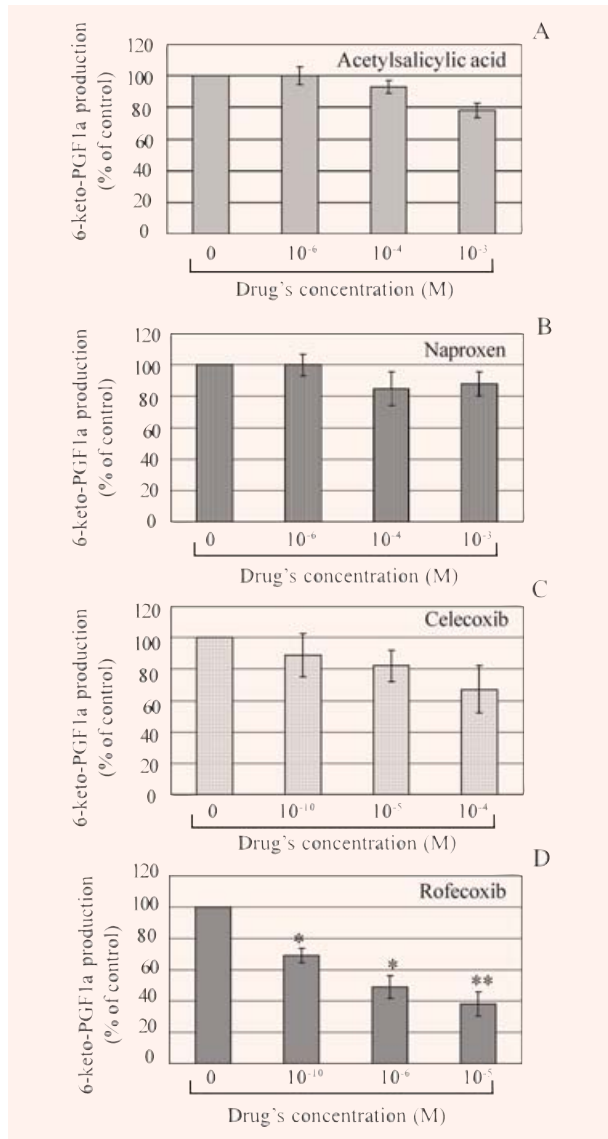We also analyzed by confocal microscopy PGIS intracellular distribution in HUVEC treated for 24 hrs

Fig. 2 Effect of non-selective NSAIDs and selective COX-2 inhibitors on PGIS activity in HUVEC. HUVE cells were treated with acetylsalicylic acid (panel **A**), naproxen (panel **B**), celecoxib (panel **C**) and rofecoxib (panel **D**) at different doses as described under Materials and Methods. After 24 hrs, the medium was changed and PGH2 (1 μM) was added for 10 min. The amount of 6-keto-PGF1α released into the cell medium was evaluated by ELISA assay and it is reported in the graphs, normalised for the cell number, as percentage of control (dose 0 of the drug). Values represents the mean ± S.E.M. of three independent experiments performed under the same conditions (n = 12). * = P < 0.05; ** = P < 0.01.
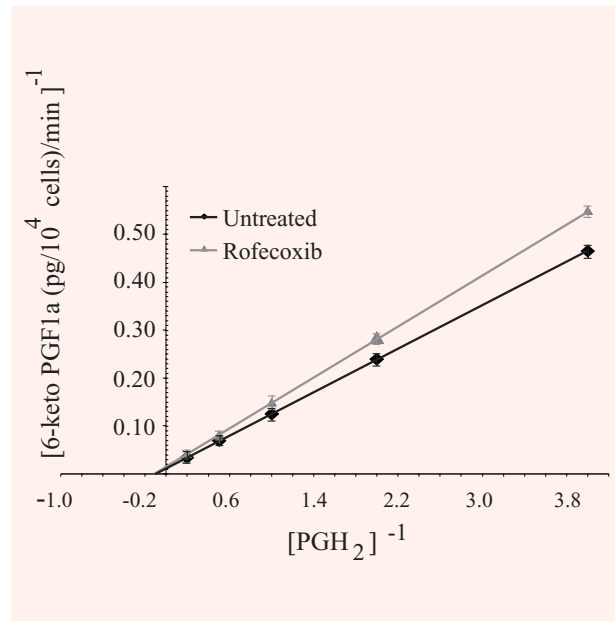

Fig. 3 Enzymatic properties of PGIS in HUVEC, in the absence or in the presence of rofecoxib. HUVE cells were untreated or treated with rofecoxib at 10^{-5} M concentration, as described under Materials and Methods. After 24 hrs, the medium was changed and the substrate PGH2 was added at different concentrations (ranging from 0.25 μM to 5 μM). The enzyme reaction was allowed to proceed for 10 min. The amount of 6-keto-PGF1α released into the cell medium was evaluated by ELISA assay. Data are represented by a Lineweaver-Burk plot as mean of three independent experiments ± SEM, (n = 12). The slope of the line, analyzed with SPSS linear regression programme, was 0.08 in the absence of rofecoxib and increased to 0.12 in the presence of rofecoxib at 10^{-5} M.

with acetylsalicylic acid, naproxen, celecoxib and rofecoxib at the higher doses previously tested by ELISA assay and Western blotting (Fig. 5). All the inhibitors tested, and in particular rofecoxib, did not cause evident changes of PGIS intracellular distribution with respect to control cells (compare panels E–H to panels A–D). In particular, the colocalization of PGIS with caveolin-1 (Cav-1), which is relevant for its activity [18], was not altered following the treatment with rofecoxib. This result indicates that the inhibition of PGIS activity caused by rofecoxib is not related to a misallocation of the enzyme leading to an impairment of its function.

Fig. 4 Effect of non-selective NSAIDs and selective COX-2 inhibitors on PGIS protein levels and mRNA expression in HUVEC. HUVE cells were treated with acetylsalicylic acid, naproxen, celecoxib and rofecoxib at different doses as described under Materials and Methods (**A** and **B**). After 24 hrs, cell lysates were prepared and analyzed by immunoblotting to detect PGIS protein levels. Lane 1: PGIS standard; lanes 2–4: samples treated with acetylsalicylic acid at different doses (10^{-3} M, 10^{-4} M, 10^{-6} M); lanes 5–7: samples treated with naproxen at different doses (10^{-3} M, 10^{-4} M, 10^{-6} M); lanes 8–10: samples treated with celecoxib at different doses (10^{-4} M, 10^{-5} M, 10^{-10} M); lanes 11–13: samples treated with rofecoxib at different doses (10^{-5} M, 10^{-6} M, 10^{-10} M); lane 14: control (untreated sample). For each sample the intensity of the band corresponding to PGIS (panel **A**) was normalized with respect to that corresponding to β-actin and results are represented in panel **B** as percentage of control. PGIS and COX-2 mRNA levels were analysed by real-time PCR (panel **C**) in HUVE cells after 24 hrs of incubation in the absence (lanes 1–2) or in the presence (lanes 3–4) of 10^{-5}M rofecoxib. No statistically significant differences in PGIS mRNA expression were detectable after rofecoxib treatment. COX-2 mRNA expression, before and after rofecoxib treatment, is also shown. Bars represent values normalized against GUSB mRNA levels. Values are the mean ± S.E.M. of three independent experiments (n = 9) performed under the same experimental conditions.
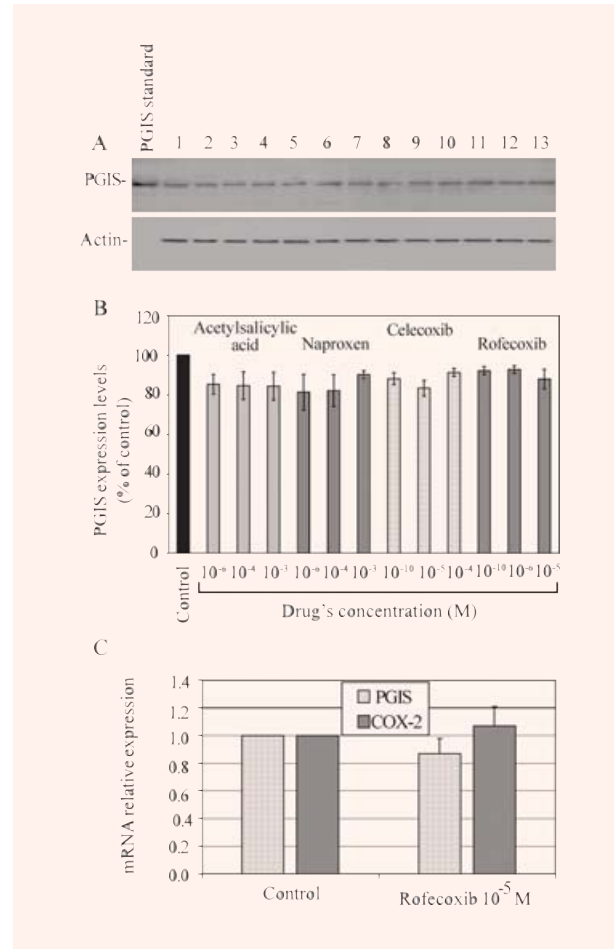
PGIS activity in bovine aortic microsomes treated with non-selective NSAIDs and selective COX-2 inhibitors

Since the purification of functionally intact PGIS results to be very difficult [21], we further investigated the effect of rofecoxib, compared to that of non-selective NSAIDs and celecoxib, by using bovine aortic microsomal (BAMs) fractions enriched in PGIS. In BAMs, COX-2 and COX-1 proteins were not detectable by Western blotting analysis (data not shown). We first observed that the inhibitory effect of rofecoxib on PGIS in BAMs was nearly absent if incubation was performed for 10–15 min before the addition of the substrate PGH2, but it significantly increased if microsomes were incubated in the presence of the drug for 1 hr at 37°C before the addition of the substrate. Under these conditions a 10^{-4} M

concentration of rofecoxib caused a 50% inhibition of PGIS activity, while celecoxib as well as non-selective NSAIDs at the same concentrations showed no significant effects (Fig. 6). Moreover, we did not observe any significant inhibitory effect for rofecoxib at lower doses (*i.e.* 10^{-5} M), which were effective on PGIS activity in HUVE cells. We hypothesized that rofecoxib needs to be chemically modified in order to inhibit PGIS activity. Thus, the weaker inhibitory effect of rofecoxib in BAMs could be due to the lack of cellular co-factors (like NADPH or GSH), relevant for the activity of enzymatic complexes able to metabolize and modify drugs (*i.e.* Cytochrome P450 or Glutathione-S-transferase). However, following addition of NADPH or GSH to the microsomal preparations, we failed to detect an increase in the inhibitory effect of rofecoxib (data not shown).

Taking together, results reported here provide evidence that rofecoxib, but not celecoxib as well as clas-
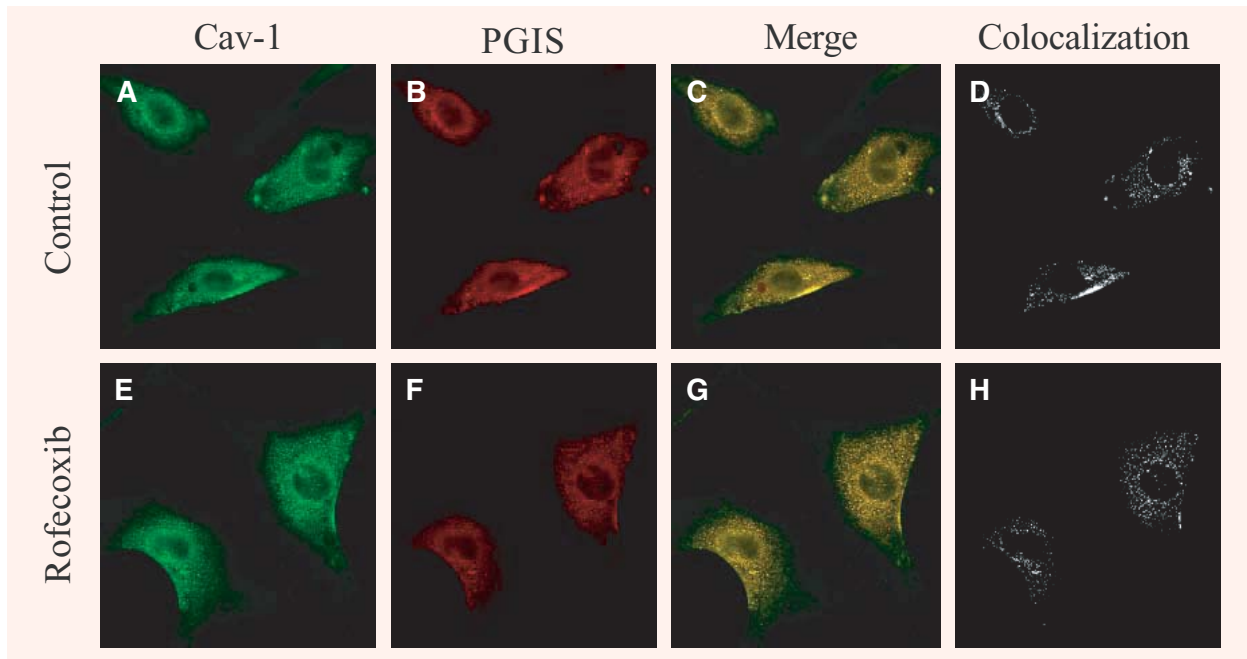
Fig. 5 Effect of non-selective NSAIDs and selective COX-2 inhibitors on PGIS intracellular distribution in HUVEC. HUVE cells, seeded on cover slides and treated with acetylsalicylic acid (10^{-3} M), naproxen (10^{-3} M), celecoxib (10^{-4} M) and rofecoxib (10^{-5} M) for 24 hrs, were stained with PGIS and Cav-1 antibodies and analyzed by confocal microscopy. The figure represents images regarding PGIS and Cav-1 distributions in control (untreated) cells (panels **A–D**) and in rofecoxib-treated cells (panels **E–H**), because results were similar for all the treatments tested. Cav-1 distribution (green signal) is represented in panels **A** and **E** while PGIS distribution (red signal) is represented in panels **B** and **F**. The merging of the two signals (yellow) is reported in panels **C** and **G**. The co-localization maps, obtained as described under Materials and Methods, are reported in panels **D** and **H**. Bar: 10 μm.

sical anti-inflammatory compounds (acetylsalicylic acid and naproxen), strongly inhibits PGIS activity in human endothelial cells. This inhibition is not due to a modification of the enzyme expression or to a modification of its subcellular localization, but it is likely due to an interaction of rofecoxib with PGIS, probably acting as a non-competitive inhibitor of the enzyme.

Discussion

Selective inhibitors of COX-2 (coxibs) have been developed starting from 1990. Preclinical and clinical studies revealed the good efficacy of celecoxib and rofecoxib in inflammation, fever and pain. Furthermore, they confirmed that selective COX-2 inhibitors have greater gastrointestinal safety and do not affect platelet aggregation. Following these results, celecoxib and rofecoxib were rapidly licensed by the Food and Drug

Administration (FDA) in 1999 for the treatment of osteoarthritis and rheumatoid arthritis [35].

In addition to the expected complications in the renal system, due to the relevance of COX-2 activity in the macula densa of the kidney [3], severe effects of COX-2 selective inhibitors on the cardiovascular system have emerged during their clinical trials. The main clinical trial designed to rigorously assess the gastrointestinal (GI) safety of Vioxx (VIGOR study) has been published in 2000 and demonstrated that a supratherapeutic dose of rofecoxib was associated with a significantly reduced risk of clinical upper GI events compared with naproxen. This study also revealed, after 9 months of clinical trial, a higher incidence of myocardial infarction among patients in the rofecoxib group, presented by the investigators as a cardioprotective effect of naproxen [30]. Following the results of the Adenomatous Polyp Prevention on Vioxx (APPROVe) trial, emerged in September 2004, Merck & Co. voluntarily withdrew Vioxx from the market. The APPROVe

study reported the cardiovascular outcomes of a long-term, randomized, placebo-controlled trial designed to determine the effects of three years of treatment with rofecoxib on the risk of recurrent neoplastic polyps in patients with a history of colorectal adenoma [31]. The study revealed that, after 18 months of treatment, rofecoxib doubled the risk of thrombotic events with respect to placebo (relative risk 1.92).

Following the rofecoxib history, several controversial studies examined the question about cardiovascular risk associated to selective COX-2 inhibitors in comparison with traditional NSAIDs. Recently, Graham and colleagues [36] analyzed the risk of serious coronary heart disease during the treatment with rofecoxib at standard or high doses in comparison with classical NSAIDs use or celecoxib use, as celecoxib was the most common alternative to rofecoxib. They reported that rofecoxib use involves a higher risk of acute myocardial infarction and sudden cardiac death if compared with celecoxib use. Furthermore, following the analysis of clinical trials and in particular the CLASS study (Celecoxib Long Term Arthritis Safety Study), celecoxib has been considered much less prone to affect the cardiovascular system and, to this date, it is still marketed [37–39]. The safety of celecoxib has not been confirmed by Solomon and collaborators [32], who found an increased cardiovascular risk associated with celecoxib treatment in two long-term clinical trials for the prevention of colorectal adenomas.

Predisposition of patients treated with coxibs to myocardial infarction has been explained by an imbalance between prostacyclin (PGI2) and thromboxane A2 (TxA2), due to the inhibition of COX-2 dependent PGI2 production by endothelial cells, without any effect on TxA2 produced by platelet COX-1 [40]. This imbalance should decrease the protective effect of PGI2 within the vasculature, potentially creating a prothrombotic state [41, 42].

Nevertheless, the absence of a clear relationship between COX-2 selectivity and the observed cardiovascular side effects suggests that other mechanisms could be involved in the cardiovascular side effects outcome. This hypothesis is in some measure corroborated by the study of Farkouh and collaborators [43] who showed that lumiracoxib, a very selective coxib, seems not to increase cardiovascular outcomes after one year of follow up.
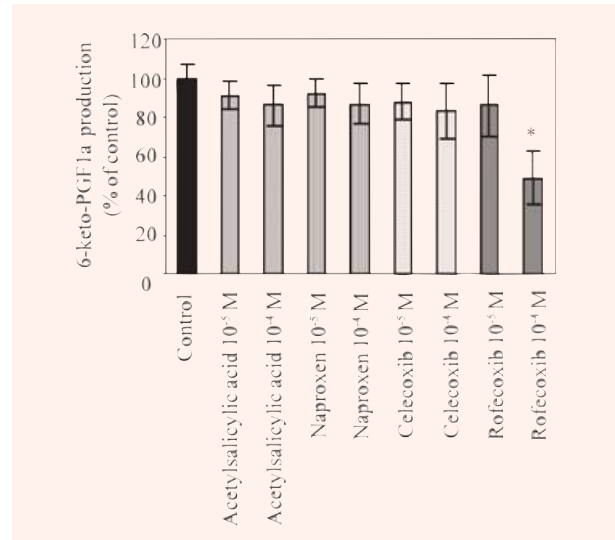

Fig. 6 Effect of non-selective NSAIDs and selective COX-2 inhibitors on PGIS activity in bovine aortic microsomes (BAMs). 2 μg of bovine aortic microsomal fractions enriched in PGIS were incubated with acetylsalicylic acid, naproxen, celecoxib and rofecoxib at different doses for 1 h at 37°C as described under Materials and Methods. After addition of the substrate PGH2 (1 μM), the reaction products were extracted, 6-keto-PGF1α production was evaluated by ELISA assay and it is reported in the graph as percentage of control (representing PGIS activity in BAMs in the absence of any treatment). Values are the mean ± S.E.M. of three independent experiments (n = 12) performed under the same experimental conditions. * = P < 0.01.

These considerations prompted us to hypothesize that rofecoxib cardiotoxicity could be explained by assuming that other molecular mechanisms, independent from COX-2 inhibition, should be involved. Data reported here strongly support the hypothesis that an inhibition of prostacyclin synthase (PGIS) activity induced by rofecoxib is implicated in its cardiovascular toxicity. In fact, we observed a significant inhibition of PGIS activity in human endothelial cells exposed to rofecoxib, even at a dose which resulted ineffective on COX-2 activity. It is important to underline that in this study we used lower NSAIDs concentrations respect to those recorded during pharmacokinetic experiments in humans. In particular, the concentration of rofecoxib in human plasma, in patients who received 25 mg in single dose, ranged from 0.05 to 1 μM, while for celecoxib, after a 200 mg single

dose, plasma concentrations ranged from 0.1 to 2.5 μM [44]. Our data demonstrate that this PGIS inhibition could be due to a direct interaction of rofecoxib with the enzyme, affecting its activity, probably by a non-competitive mechanism. This interaction could involve the heme distal pocket of PGIS, which is known to be fairly adaptable to ligands of various structures, like the PGIS inhibitors clotrimazole and tranylcypromine previously described [45, 46]. The binding of these inhibitors to PGIS requires a hydrophobic interaction within the enzyme pocket, which may occur for rofecoxib too, explaining the long incubation time necessary to achieve an inhibition during experiments performed on PGIS-enriched microsomal fractions.

A compensatory mechanism increasing COX-1 levels following COX-2 inhibition has been proposed [2]. If it is true, it is very likely that enough PGI2 is still produced to protect the cardiovascular system when endothelial cells are exposed to COX-2 selective inhibition and PGIS remains fully active. Thus, it is plausible that PGI2 synthesis, in the presence of rofecoxib and especially when it is chronically assumed, becomes inadequate to protect the cardiovascular system. This PGIS inhibition may explain why rofecoxib-induced cardiovascular side effects raised earlier during the treatment with respect to those observed during celecoxib assumption [32, 37–39].

Even if rofecoxib seems to be the unique anti-inflammatory drug capable of PGIS inhibition so far discovered, we can not exclude that other selective COX-2 inhibitors such as valdecoxib, taken off from the market due to the increased risk of cardiovascular events, may inhibit PGIS. For this reason, we strongly recommend that in future novel coxibs should be tested *in vitro* and *in vivo* as possible PGIS inhibitors.

Acknowledgments

References

1. **Smith WL, DeWitt DL, Garavito RM.** Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem.* 2000; 69: 145–82.

2. **Wang H, Ma WG, Tejada L, Zhang H, Morrow JD, Das SK, Dey SK.** Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic make-up. *J Biol Chem.* 2004; 279: 10649–58.

3. **Nasrallah R, Hébert RL.** Prostacyclin signaling in the kidney: implication for health and disease. *Am J Physiol Renal Physiol.* 2005; 289: 235–46.

4. **Prescott SM, Fitzpatrick FA.** Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta.* 2000; 1470: 69–78.

5. **Brown JR, DuBois RN.** COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol.* 2005; 23: 2840–55.

6. **Bunting S, Gryglewski R, Moncada S, Vane JR.** Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac ateries and inhibits platelet aggregation. *Prostaglandins.* 1976; 12: 897–913.

7. **Moncada S, Gryglewski R, Bunting S, Vane JR.** An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature.* 1976; 263: 663–5.

8. **Weksler BB, Marcus AJ, Jaffe EA.** Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. *Proc Natl Acad Sci USA.* 1977; 74: 3922–6.

9. **Tomasi V, Meringolo C, Bartolini G, Orlandi M.** Biosynthesis of prostacyclin in rat liver endothelial cells and its control by prostaglandin E2. *Nature.* 1978; 237: 670–1.

10. **Tomasi V, Meringolo C, Bartolini G, Orlandi M.** Effect of prostaglandin E2 on prostacyclin production by endothelial cells. *Nature.* 1979; 274: 48–9.

11. **Negishi M, Sugimoto, Ichikawa A.** Molecular mechanisms of diverse actions of prostanoid receptors. *Biochim Biophys Acta.* 1995; 1259: 109–19.

12. **Forman BM, Chen J, Evans RM.** Hypolipidemic drugs, polyunsatured fatty acids and eicosanoids are ligands for peroxisome proliferator-activated receptors α and β. *Proc Natl Acad Sci USA.*1997; 94: 4312–7.

13. **Geraci MW, Gao B, Shepherd DC, Moore MD, Westcott JY, Fagan KA, Alger LA, Tuder RM, Voelkel NF.** Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension. *J Clin Invest.* 1999; 103: 1509–15.

14. **Iwai N, Katsuya T, Ishikawa K, Mannami T, Ogata J, Higaki J, Ogihara T, Tanabe T, Baba S.** Human prostacyclin synthase gene and hypertension: The Suita Study. *Circulation.* 1999; 100: 2231–6.

15. **Lim H, Gupta RA, Ma W, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK.**

Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse *via* PPAR γ. *Genes Dev.* 1999; 13: 1561–74.

16. **Spisni E, Tomasi V.** Involvement of prostanoids in angiogenesis. In: Bicknell R, Lewis CE, Ferrara N, editors. *Tumour angiogenesis.* Oxford University Press; 1997. p. 291–300.

17. **Tomasi V, Spisni E, Griffoni C, Guarnieri T.** Caveolae, caveolar enzymes and angiogenesis. *Current Topics in Biochemical Research.* 2000; 3: 81–90.

18. **Spisni E, Griffoni C, Santi S, Riccio M, Marulli R, Bartolini G, Toni M, Ullrich V, Tomasi V.** Colocalization prostacyclin (PGI2) synthase- caveolin-1 in endothelial cells and new roles for PGI2 in angiogenesis. *Exp Cell Res.* 2001; 266: 31–43.

19. **Massimino ML, Griffoni C, Spisni E, Toni M, Tomasi V.** Involvement of caveolae and caveolae-like domains in signalling cell survival and angiogenesis. *Cell Signall.* 2002; 14: 93–8.

20. **Hao CM, Redha R, Morrow J, Breyer MD.** Peroxisome proliferator-activated receptor delta activation promotes cell survival following hypertonic stress. *J Biol Chem.* 2002; 277: 21341–5.

21. **Wu KK, Liou J-Y.** Cellular and molecular biology of prostacyclin synthase. *Biochem Biophys Res Commun.* 2005; 338: 45–52.

22. **Spisni E, Bartolini G, Orlandi M, Belletti B, Santi S, Tomasi V.** Prostacyclin (PGI2) synthase is a constitutively expressed enzyme in human endothelial cells. *Exp Cell Res.* 1995; 219: 507–13.

23. **Liou JY, Deng WG, Gilroy DW, Shyue SK, Wu KK.** Co-localization and interaction of cyclooxygenase-2 with caveolin-1 in human fibroblasts. *J Biol Chem.* 2001; 276: 34975–82.

24. **Kim SF, Huri DA, Snyder SH.** Inducible nitric oxide synthase binds, s- nitrosylates, and activates cyclooxygenase-2. *Science.* 2005; 310: 1966–70.

25. **Gibson LL, Hahner L, Osborne-Lawrence S, German Z, Wu KK, Chambliss KL, Shaul PW.** Molecular basis of estrogen-induced cyclooxygenase type 1 upregulated in endothelial cells. *Circulation Res.* 2005; 96: 518–25.

26. **Doré M, Chevalier S, Sirois J.** Estrogen-dependent induction of cyclooxygenase-2 in the canine prostate *in vivo*. *Vet Pathol.* 2005; 42: 100–3.

27. **FitzGerald GA, Patrono C.** The coxibs, selective inhibitors of cyclooxygenase-2. *N Engl J Med.* 2001; 345: 433–42.

28. **Hankey GJ, Eikelboom JW.** Aspirin resistance. *Lancet.* 2006; 367: 606–17.

29. **Warner TD, Giuliano F, Vojnovic I, Bukasa A, Mitchell JA, Vane JR.** Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. *Proc Natl Acad Sci USA.* 1999; 96: 7563–8.

30. **Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, Day R, Ferraz MB, Hawkey CJ, Hochberg MC, Kvien TK, Schnitzer TJ, VIGOR Study Group.** Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med.* 2000; 343: 1520–8.

31. **Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A, Konstam MA, Baron JA.** Adenomatous Polyp Prevention on Vioxx (APPROVe) Trial Investigators. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med.* 2005; 352: 1092–102.

32. **Solomon SD, Pfeffer MA, McMurray JJ, Fowler R, Finn P, Levin B, Eagle C, Hawk E, Lechuga M, Zauber AG, Bertagnolli MM, Arber N, Wittes J.** APC and PreSAP Trial Investigators. Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas. *Circulation.* 2006; 114: 1028–35.

33. **Jamali F, Sattari S.** High performance liquid chromatografic determination of cyclooxygenase II inhibitor rofecoxib in rat and human plasma. *J Pharm Pharm Sci.* 2000; 3: 312–7.

34. **Salmon JA, Flower RJ.** Preparation and assay of prostacyclin synthase. *Methods Enzymol.* 1982; 86: 91–9.

35. **Flower RJ.** The development of COX2 inhibitors. *Nat Rev Drug Discov.* 2003; 2: 179–91.

36. **Graham DJ, Campen D, Hui R, Spence M, Cheetham C, Levy G, Shoor S, Ray WA.** Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non selective non-steroidal anti-inflammatory drugs: nested case-control study. *Lancet.* 2005; 365: 475–81.

37. **Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS.** Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA.* 2000; 284: 1247–55.

38. **Brophy JM.** Celecoxib and cardiovascular risks. *Expert Opin Drug Saf.* 2005; 4: 1005–15.

39. **King VL, Trivedi D, Gitlin JM, Loftin CD.** Selective cyclo-oxygenase-2 inhibition with celecoxib decreases

angiotensin II-induced abdominal aortic aneurysm formation in mice. *Arterioscler Thromb Vasc Biol.* 2006; 26: 1137–43.

40. **FitzGerald GA.** Coxib and cardiovascular disease. *N Engl J Med.* 2004; 351: 1709–11.

41. **Bishop-Bailey D, Mitchell JA, Warner TD.** COX-2 in cardiovascular disease. *Arterioscler Thromb Vasc Biol.* 2006; 26: 956–8.

42. **Klumpp G, Schildknecht S, Nastainczyk W, Ullrich V, Bachschmid M.** Prostacyclin in the cardiovascular system: new aspects and open questions. *Pharmacol Rep.* 2005; 57: 120–6.

43. **Farkouh ME, Kirshner H, Harrington RA, Ruland S, Verheugt FW, Schnitzer TJ, Burmester GR, Mysler E, Hochberg MC, Doherty M, Ehrsam E, Gitton X, Krammer G, Mellein B, Gimona A, Matchaba P, Hawkey CJ, Chesebro JH.** TARGET Study Group. Comparison of lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), cardiovascular outcomes: randomised controlled trial. *Lancet.* 2004; 364: 675–84.

44. **Hamama AK, Ray J, Day RO, Brien JA.** Simultaneous Determination of rofecoxib and celecoxib in human plasma by high-performance liquid chromatography. *J Chromatogr Sci.* 2005; 43: 351–4.

45. **Yeh HC, Hsu PY, Wang JS, Tsai AL, Wang LH.** Characterization of heme environment and mechanism of peroxide bond cleavage in human prostacyclin synthase. *Biochim Biophys Acta.* 2005; 1738: 121–32.

46. **Wada M, Yokoyama C, Hatae T, Shimonishi M, Nakamura M, Imai Y, Ullrich V, Tanabe T.** Purification and characterization of recombinant human prostacyclin synthase. *J Biochem.* 2004; 135: 455–63.