Life Sciences 88 (2011) 24–30

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Inducible COX-2 dominates over COX-1 in prostacyclin biosynthesis: Mechanisms of COX-2 inhibitor risk to heart disease

Cheng-Huai Ruan [a,*], Shui-Ping So [b], Ke-He Ruan [b,*]

[a] Department of Internal Medicine, New York Hospital Medical Center of Queens/Weil Cornell Medical College Affiliated Hospital, Flushing, NY 11355, United States
[b] Center for Experimental Therapeutics and PharmacoInformatics, University of Houston, Houston, TX 77204, United States

ARTICLE INFO

Article history:
Received 13 May 2010
Accepted 15 October 2010

Keywords:
Cyclooxygenase-1 (COX-1)
COX-2
COX-2 inhibitor
Arachidonic acid (AA)
Prostacyclin or prostaglandin I_2 (PGI_2)
PGI_2 synthase (PGIS) and FRET

ABSTRACT

Aim: Our aim is to understand the molecular mechanisms of the selective nonsteroidal anti-inflammatory drugs (NSAID), cyclooxygenase-2 (COX-2) inhibitors', higher "priority" to reduce synthesis of the vascular protector, prostacyclin (PGI2), compared to that of nonselective NSAIDs.
Main methods: COX-1 or COX-2 was co-expressed with PGI2 synthase (PGIS) in COS-7 cells. The Km and initial velocity (½t Vmax) of the coupling reaction between COX-1 and COX-2 to PGIS were established. The experiment was further confirmed by a kinetics study using hybrid enzymes linking COX-1 or COX-2 to PGIS. Finally, COX-1 or COX-2 and PGIS were respectively fused to red (RFP) and cyanic (CFP) fluorescence proteins, and co-expressed in cells. The distances between COXs and PGIS were compared by FRET.
Key findings: The Km for converting arachidonic acid (AA) to PGI2 by COX-2 coupled to PGIS is ~2.0 μM; however, it was 3-fold more (~6.0 μM) for COX-1 coupled to PGIS. The Km and ½t Vmax for COX-2 linked to PGIS were ~2.0 μM and 20 s, respectively, which were 2–5 folds faster than that of COX-1 linked to PGIS. The FRET study found that the distance between COX-2-RFP and PGIS–CFP is shorter than that between COX-1-RFP and PGIS–CFP.
Significance: The study provided strong evidence suggesting that the low Km, faster ½t Vmax, and closer distance are the basis for COX-2 dominance over COX-1 (coupled to PGIS) in PGI2 synthesis, and further demonstrated the mechanisms of selective COX-2 inhibitors with higher potential to reduce synthesis of the vascular protector, PGI2.

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Introduction

Arachidonic acid (AA) can be metabolized into biologically active prostanoids including prostacyclin (PGI2) and thromboxane A2 (TXA2) through the cyclooxygenase (COX)-1 (COX-1) and -2 (COX-2) pathways (Majerus 1983; Pace-Asciak and Smith 1983; Samuelsson et al. 1978; Smith 1986; Funk 2001; Ruan 2004; Ruan and Dogné 2006; Dogné et al. 2006). The synthesized TXA2 mediates platelet aggregation and vascular constriction, thus promoting myocardial infarction and stroke (Needleman et al. 1986; Granstrom et al. 1982; Patrono et al. 1990). Conversely, PGI2 counters these effects and is considered a major vascular protective mediator. In the cells, the detailed steps for the biosynthesis of these two mediators involve catalyzing AA into an intermediate, prostaglandin G2 (PGG2), and PGH2 by COX-1 or COX-2 and further isomerizing PGH2 to TXA2 or PGI2 by downstream individual TXA2 and PGI2 synthases, respectively (Majerus 1983; Pace-Asciak and Smith 1983; Samuelsson et al. 1978; Smith 1986; Funk 2001; Ruan 2004; Ruan and Dogné 2006; Dogné et al. 2006). PGI2 synthesized through the COX-1 and COX-2 pathways is important for protection against blood clots and vasoconstriction in many organs including the heart, kidneys, and brain. Reduced PGI2 production is associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs), and has a potential to cause organ ischemia. This particularly occurs during the use of selective COX-2 inhibitors (Vane 2002). The absence of COX-2 in platelets could increase the ratio of TXA2/PGI2 with the use of COX-2 inhibitors. This increased risk for cardiovascular events in clinical trials has been proposed (Martinez-Gonzalez and Badimon 2007). However, the molecular mechanisms behind the COX-2 inhibitors' greater reduction in PGI2 production than that of nonselective NSAIDs, which inhibit both COX-1 and COX-2, have not been settled yet. The majority of explanations have come from clinical trials and speculations. For example, Vane hypothesized a model, in which the specific inhibition of COX-2 activity (using a COX-2 inhibitor) would result in more AA that would be available for conversion into TXA2 (through COX-1 coupling to thromboxane A2 synthase (TXAS)), which promotes heart disease (Vane 2002). This

* Corresponding authors. Cheng-Huai Ruan is to be contacted at Tel.: +1 718 661 7621; fax: +1 713 743 1884. Ke-He Ruan, The Center for Experimental Therapeutics and PharmacoInformatics, Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Room 521 Science and Research 2 Building, Houston, TX 77204-5037, United States. Tel.: +1 713 743 1771; fax: +1 713 743 1884.
E-mail addresses: chr9062@nyp.org (C.-H. Ruan), khruan@uh.edu (K.-H. Ruan).

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doi:10.1016/j.lfs.2010.10.017

implies that COX-2 has priority over COX-1 in terms of acting upon AA. Finding answers behind this "priority" is a key step toward uncovering the molecular mechanisms of COX-2 inhibition, a risk to heart disease and further validating Vane's model. In this study, we are focusing on the characterization of COX enzyme kinetics and the topological coordination for the coupling between either COX-1 or COX-2 with PGI2 synthase (PGIS) in the biosynthesis of PGI2 in live cells. The study has demonstrated that COX-2's coupling to PGIS, and in turn the conversion of AA into PGI2, has a lower Km value and a faster initial reaction velocity in comparison to that of COX-1. The topological distance of PGIS is closer to COX-2 than that of COX-1 in live cells. These two factors could be used to further speculate that in the presence of COX-1, COX-2, and PGIS, COX-2 could be preferred over COX-1 in coupling to PGIS, and thus dominate PGI2 biosynthesis. This could be the mechanism of specific COX-2 inhibitors having stronger capability to reduce PGI2 production and promote heart disease in clinical trials. Our study supports Vane's model, in which selective COX-2 inhibitors have more potential risks than that of nonselective NSAIDs in regard to promoting heart disease.

Experimental procedures

Materials

COS-7 cell line was purchased from ATCC (Manassas, VA). Medium for culturing the cell lines was purchased from Invitrogen. [^{14}C]-Arachidonic acid (AA) was purchased from Amersham Biosciences. Other reagents were from Sigma.

Molecular cloning

For the wild type enzymes, the cDNAs of human COX-1, COX-2 and PGIS were cloned by a polymerase chain reaction (PCR) approach and then subcloned into a pcDNA3.1 vector with a CMV promoter (Deng et al. 2002, 2003; Ruan et al. 2005). The cloning of the hybrid enzymes, COX-1-10aa-PGIS and COX-2-10aa-PGIS were performed by the approaches described previously (Ruan et al. 2006, 2008a,b). Briefly, the cDNA of human COX-1 or COX-2 was linked to a 10 amino acid transmembrane (TM) linker connected to human PGIS, and then subcloned into pcDNA3.1 vector (Ruan et al. 2006, 2008a,b). For preparation of the enzymes tagged with fluorescent proteins, the cDNA of C-terminus of COX-1 or COX-2 was linked to the N-terminus of RFP, and the C-terminus of PGIS was linked to the N-terminal CFP, as was performed by PCR approach to form the COX-1- or COX-2-RFP and PGIS–CFP hybrid enzymes. The COX-1- or COX-2-RFP was further linked to the PGIS–CFP by PCR and ligation approaches.

Co-expression of the recombinant proteins in COS-7 Cells

The recombinant synthases were expressed in COS-7 cells as described (Deng et al. 2003; Ruan et al. 2005). Briefly, the cells were cultured in a 100-mm cell culture dish with high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. The cells were then transfected with a purified cDNA of the recombinant protein by the Lipofectamine 2000 method (Ruan et al. 2006) following the manufacturer's instructions (Invitrogen). Approximately 48 h after transfection, the cells were used for fluorescence microscopy, enzyme assays, and Western blot analysis. For the co-transfection, a 3:2 ratio of the cDNAs of COX-2 or COX-1 and PGIS was used. The ratio used was based on the optimized co-expression of the two enzymes in the cells.

Electrophoresis and Western blot

The cells were sonicated in PBS and then centrifuged at 17,000 ×*g* for 30 min at 4 °C. The cell pellets containing microsomes were solubilized using a sample buffer containing 1% SDS, and separated by 7–10% (w/v) SDS-PAGE under denaturing conditions and then transferred to a nitrocellulose membrane. Bands recognized by specific primary antibodies against human COX-1, COX-2 and PGIS were visualized with horseradish peroxidase-conjugated secondary antibody and chromogenic peroxidase substrates (Ruan et al. 2006). The intensities of the bands in the immunoblot were used to normalize the enzyme activities as well.

Enzyme activity determination for COX coupled to PGIS using the high-performance liquid chromatography (HPLC)-scintillation analysis method (Ruan et al. 2006)

To determine the activity of the enzyme that converted AA into PGI2, the cells were incubated with different concentrations of [^{14}C]-AA (0.3–60 μM) in a total reaction volume of 100 μL. After a 0.5–5 min incubation, the reaction was terminated by adding 200 μL of the solvent containing 0.1% acetic acid and 35% acetonitrile (solvent A). After centrifugation (17,000 ×*g* for 5 min), the supernatant was injected into a reverse phase C18 column (Varian Microsorb-MV 100-5, 4.5 × 250 mm) using the solvent A with a gradient from 35 to 100% of acetonitrile for 45 min at a flow-rate of 1.0 mL/min. The [^{14}C]-labeled AA metabolites, including [^{14}C]-6-keto-PGF1α (degraded PGI2) were monitored directly by a flow scintillation analyzer (Packard 150TR). The peak and the relative amount of [^{14}C]-6-keto-PGF1α converted from [^{14}C]-AA were confirmed and calibrated by 6-keto-PGF1α standards using the enzyme immunoassay described (Ruan et al. 2006).

Fluorescence microscopy and immunofluorescence staining

The cultured cells expressing COX-RFP and PGIS–CFP were directly observed by fluorescence microscopy using corresponding wavelengths of excitation and emission for the red and cyanic colors. For immunofluorescence microscopy, the transfected cells grown on a cover glass were washed with PBS and then incubated with 3.7% formaldehyde for 10 min. The cells were then blocked for 20 min before being incubated with 1% saponin for 20 min. This was followed by the addition of the primary antibody (10 μg/mL of affinity-purified anti- human COX-1, COX-2 or PGIS antibody) in the presence of 0.25% saponin and 10% goat serum for 1 h. After washing with PBS, the cells were incubated with the secondary antibodies labeled with FITC- or Rhodamine, and then viewed under an Olympus epifluorescence microscope (Ruan et al., 2006, 2008b).

FRET experiments

The fluorescence images of the live cultured cells expressing the FP-fused protein(s) were captured by the Olympus epifluorescence microscope using three sets of filters: for CFP (Ex440, m480), for RFP (Ex525, m580) and for FRET (Ex440, m580).

Results

Co-expression of COX-1 or COX-2 with PGIS in COS-7 cells

The previously cloned cDNAs of human COX-1, COX-2 and PGIS were subcloned into the mammalian expression vector, pcDNA3.1. COS-7 cells were used to co-express COX-1 or COX-2 with PGIS by gene transferring approach (Deng et al. 2002, 2003; Ruan et al. 2005). After 48-h of the transfection, the cells co-expressing the COX-2 or COX-1 with PGIS (Ruan et al. 2006) were confirmed by Western blot analysis using the antibodies against human COX-1, COX-2 and PGIS (Fig. 1). The correct molecular mass for the COX-1 (MW: 70 kDa) or COX-2 (MW: 72 kDa) co-expressed with PGIS (MW: 56 kDa) in the cells were shown (Fig. 1). The protein amounts loaded in the Western

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