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Biochemical Pharmacology 67 (2004) 245–254
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

COX-2-mediated PGD_2 synthesis regulates phosphatidylcholine biosynthesis in rat renal papillary tissue

María Fernández-Tome, Leticia Kraemer, Sebastián Cembal Federman, Nicolás Favale, Emir Speziale, Norma Sterin-Speziale*

Department of Biological Sciences, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, IQUIFIB-CONICET, Junin, 1113 Buenos Aires, Argentina

Received 2 June 2003; accepted 26 August 2003

Abstract

Phosphatidylcholine (PC) is the major membrane phospholipid in mammalian cells. Previous works from our laboratory demonstrated a close metabolic relationship between the maintenance of PC biosynthesis and the prostaglandins endogenously synthesized by cyclooxygenase (COX) in rat renal papilla. In the present work, we studied the COX isoform involved in papillary PC biosynthesis regulation. The incorporation of [methyl-^{3}H]choline and [^{32}P]orthophosphate to PC was determined in the absence and presence of SC-560 and NS-398, COX-1 and COX-2 specific inhibitors. PC synthesis was highly sensitive to COX-2 inhibition, while COX-1 inhibition only reduced PC synthesis at high SC-560 concentration. The analysis of choline-containing metabolites showed that COX-2 inhibition affected the formation of CDP-choline intermediary. The evaluation of PC biosynthetic enzymes revealed that microsomal, as well as nuclear, CTP:phosphocholine cytidylyltransferase (CCT), and nuclear-CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CTP) activities were affected by COX-2 inhibition. The addition of exogenous prostaglandin D_2 (PGD_2) restored nuclear-CCT and -CPT activities but not microsomal CCT. Papillary synthesis of PGD_2 was only detected in nuclear fraction where it was blocked by COX-2 inhibitor NS-398, but not by COX-1 inhibitor. All together, the present results demonstrated that COX-2-mediated PGD_2 synthesis is a PC biosynthesis regulator in rat renal papilla. Considering the importance of the maintenance of PC biosynthesis for the preservation of cell membrane homeostasis to ensure cell viability, and the extensive use of COX-2 inhibitors in therapeutics, the present results could have great pharmacological implications, and can constitute a biochemical explanation for the nephrotoxic effect of non-steroidal anti-inflammatory drugs.

Keywords: Renal papilla; Phosphatidylcholine (PC) biosynthesis; Cytidylyltransferase (CCT); Cholinephosphotransferase (CPT); Cyclooxygenase-2 (COX-2); Prostaglandin D_2 (PGD_2) synthesis

1. Introduction

Phospholipids are key molecules for cellular life. First, they form the lipid bilayer that determine the structural matrix of biomembranes [1]; second, they regulate the function of numerous membrane-bound proteins [2,3]; third, they contribute to membrane biophysical properties [4], and finally, they play a principal role in signal transduction pathways [5,6]. Because of the remarkable importance of phospholipid functions [7], it is crucial for cells to preserve membrane phospholipid content and composition, thus safeguarding the structural and functional integrity of their membranes.

Membrane homeostasis is maintained by a synchronized cycle of phospholipid degradation and *de novo* synthesis (turnover), through the coordinated actions of phospholipases and phospholipid biosynthetic enzymes, respectively [8–10]. As a consequence of phospholipid degradation, second messengers are generated and a decrease in biomembrane phospholipid concentration occurs. In order to diminish the intracellular concentration of bioactive

* Corresponding author. Tel.: +54-11-49648238; fax: +54-11-49625457.
E-mail addresses: fertome@mail.retina.ar (M. Fernández-Tome), speziale@huemul.ffyb.uba.ar (N. Sterin-Speziale).

Abbreviations: PC, phosphatidylcholine; CK, choline kinase; CCT, CTP, phosphocholine cytidylyltransferase; CPT, choline:1,2-diacylglycerol cholinephosphotransferase; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 1; PGs, prostaglandins; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

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doi:10.1016/j.bcp.2003.08.028

molecules, as well as to restore membrane phospholipid concentration, phospholipid biosynthesis is activated [8]. As a result of this metabolic cycle, the adequate phospholipid renewal in biological membranes occurs.

The papilla is the zone of the kidney with the most active phospholipid turnover [11]. Such ability of papillary tissue of renewing membrane phospholipids seems to be the mechanism by which cells maintain their membrane homeostasis in pathological situations. We have demonstrated that in $HgCl_2$-induced experimental acute renal failure, the papilla shows a rise in its phospholipid biosynthesis as a protective mechanism against the toxic agent [12]. A selective increase in papillary phospholipid biosynthesis is also present in rat renal papillary cells in streptozotocin-induced diabetes mellitus [13]. In both cases, the increased rate of membrane phospholipid turnover was considered a protective mechanism to maintain cellular membrane integrity. Physiologically, renal papilla is under non-favorable environment since it is submitted to the highest interstitial osmolality and to a high concentration of detoxification products [14]. In such adverse surroundings, papillary cells need to maintain membrane homeostasis; therefore, an efficient and effective phospholipid renewal is necessary to conserve cellular function integrity. It has been proposed that the protection mechanism by which cells survive in such conditions is the intracellular accumulation of osmolytes [15]. However, several experimental evidences suggest that protection is also mediated by COX-mediated prostaglandin synthesis [16]. In agreement with these reports, we have demonstrated that "*in vivo*" short- and long-term treatments with indomethacin, a potent prostaglandin synthesis inhibitor, caused serious alterations in membrane phospholipid composition [17]. In "*in vitro*" experiments, we have shown that indomethacin causes a concentration-dependent decrease in PC biosynthesis, which was completely reverted by the addition of exogenous prostaglandins, being PGD_2 the prostaglandin most tightly implicated, exerting its action by modulating the enzymes of PC biosynthetic pathway [18,19].

Three COX isoforms have been reported: COX-1, COX-2, and COX-3 [20,21]. They possess similar catalytic properties but they differ in their primary structure, in their sensitivity to pharmacological agents and in the pattern of expression [21,22]. COX-1 and COX-2 expression has been reported in mammalian kidney [23]. Under physiological conditions, renal papillary prostaglandin synthesis is mainly attributed to COX-1 enzyme, which is abundantly and constitutively expressed in papillary collecting ducts and has been proposed as a "housekeeping enzyme" since it appears to be responsible for the production of PGs that mediate the regulation of vascular tone, salt and water homeostasis, and are involved in the modulation of renal hormonal action [23]. COX-2 has been suggested as a "survival gene," playing a central role in cell cytoprotection and viability, as occurs in cultures of inner medullary collecting duct cells submitted to osmotic stress that respond by increasing COX-2 activity [15,24,25]. Typically considered an inducible isoform, COX-2 expression is rapidly induced after water deprivation [26], hypertonic stress conditions [27] and stimulation with pro-inflammatory agents in renal medullary cell cultures [25]. Constitutive expression of COX-2 isoenzyme has also been reported in renal papillary structures [23], but its physiological significance remains unclear.

Considering the importance of phospholipid renewal for preserving membrane homeostasis to ensure cell structure and viability, and our previous findings that indicate that phospholipid renewal is maintained by prostaglandins and the possible role of COX-2 as cell cytoprotector, we have now evaluated which COX isoform is involved in the prostaglandin synthesis that maintains PC biosynthesis. Our results demonstrate that papillary PC biosynthesis depends on COX-2-dependent PGD_2 synthesis, which operates in the final steps of PC biosynthetic pathway occurring at nuclear compartment.

2. Materials and methods

2.1. Materials

Carrier free [^{32}P]orthophosphate (specific activity 25 mCi/mmol) and [methyl-^{14}C]CDP-choline (specific activity 52.0 mCi/mmol) were purchased from Amersham Pharmacia Biotech Argentina SA (Buenos Aires); [methyl-^{3}H]choline (specific activity 51.0 mCi/mmol), [1-^{14}C]arachidonic acid (specific activity 52.8 mCi/mmol) and [methyl-^{14}C]phosphorylcholine (specific activity 50.0 mCi/mmol) were obtained from New England Nuclear; X-ray film for autoradiography from Eastman Kodak Co. TLC silica gel plates were obtained from Merck. Indomethacin, enzyme substrates and cofactors, and prostaglandin standards were purchased from Sigma Chemical Co. SC-560 and NS-398 were obtained from Cayman Chemical Co. All other reagents and chemicals were of analytical grade (Merck or Mallinckrodt) and purchased from local commercial suppliers.

2.2. Preparation of kidney tissue

Male Wistar rats (body weight: 250–300 g) were sacrificed by decapitation, and both kidneys removed and kept in an ice-cold Krebs-Ringer buffer, pH 7.4, containing 5.5 mM glucose. The medium was gassed with 95% O_2/5% CO_2. Each kidney was cut in halves through the pelvis along its longitudinal axis and the papilla (whitish inner medulla) was isolated by scissors and scalpel dissection.

2.3. COX activity in homogenates

Papillary samples (10 mg of wet weight tissue) were homogenized in 25 mM Tris–HCl, pH 8, containing

10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF. Homogenate aliquots of 100 μg of protein were incubated at 37° with 100 μM of [1-^{14}C]AA, 1 mM GSH and 1 mM epinephrine for 30 min, both in the absence and in the presence of different concentrations of indomethacin (non-selective COX inhibitors), and SC-560 or NS-398 (COX-1 and COX-2 specific inhibitors, respectively) [28]. Reaction was stopped on ice by adding 1 M citric acid up to pH 3, and prostaglandins extracted by adding 1 mL of chloroform [29]. The organic phase was removed and the aqueous phase was washed twice with one part of chloroform. The chloroform phases were pooled and dried at 25° in N_2 atmosphere. The extracts were redissolved in chloroform/methanol (2:1 v/v) and applied onto silica gel G precoated thin-layer chromatography plates. Prostaglandins and appropriate standards were separated by utilizing the upper phase of a mixture containing isooctane/ethylacetate/acetic acid/water (66:30:12:60 v/v/v/v) as solvent system [30]. Specific areas corresponding to each prostaglandin were visualized by autoradiography, scrapped off and quantified by liquid scintillation counting.

2.4. Subcellular membrane preparation

Papillary slices (100–200 mg of wet weight tissue) were incubated for 90 min at 37° both in the absence and in the presence of SC-560 or NS-398. Following incubation, samples were immediately homogenized in 10 vol. of a solution 0.25 M sucrose containing 25 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 2 mM EGTA, and successively centrifuged at 860 *g* 10 min, 8000 *g* 20 min, and 105,000 *g* 60 min. The pellet obtained at 105,000 *g* was used as microsomal fraction. To obtain enriched-nuclei fraction, the pellet obtained from the first sedimentation was resuspended in 3.8 vol. of a 2.4 M sucrose solution containing 25 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$ and 2 mM EGTA, and centrifuged at 50,000 *g* for 60 min; the resulting pellet was used as nuclear fraction (Nu) [31,32]. The purity of nuclei was assessed by microscopy and by the measurement of glucose 6-phosphatase and 5′ nucleosidase activities, to evaluate endoplasmic reticulum (ER) and plasma membrane contamination, respectively [33]. These assays showed that 2.4 M sucrose pellets were highly enriched in nuclei. The activity of 5′ nucleosidase was 40 ± 13 nmol of inorganic phosphorous/mg and 1080 ± 198 nmol of inorganic phosphorous/mg protein in 60 min in Nu and microsomal membranes, respectively. Glucose 6-phosphatase activity was 210 ± 106 nmol of inorganic phosphorous/mg and 1840 ± 215 nmol of inorganic phosphorous/mg protein in 60 min in nuclei and microsomes, respectively, corresponding to the fact that the outer nuclear membrane is contiguous with the ER. The different subcellular fractions were also obtained from untreated papillary tissue in order to study the possible direct effect of the inhibitors on the PC biosynthetic enzymes.

2.5. Phosphatidylcholine biosynthesis assays

Biosynthetic pathway was evaluated by measuring PC labeling with different precursors: [^{32}P]orthophosphate and [methyl-^{3}H]choline. Tissue slices (5 mg wet weight) were collected in 200 μL of ice-cold Tris–HCl buffer, pH 7.4, containing 5.5 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$ and 1 mM $CaCl_2$, and incubated at 37° with radiolabeled precursors in a metabolic shaking bath in an atmosphere of 95% O_2/5% CO_2. Time course experiments were performed but the results shown in the figures correspond to the time of the steady-state equilibrium (70 min for ^{32}P and 90 min for [^{3}H]choline). In order to study the relationship between the activities of COX and PC biosynthesis, different concentrations (10^{-9} to 10^{-4} M) of SC-560 and NS-398 (COX-1 and COX-2 specific inhibitors, respectively) were added to the incubation medium in another set of experiments. After treatments, PC analysis was carried out as previously described [12]. Briefly, incubations were stopped on ice and tissue samples rapidly homogenized. Lipids were extracted by adding four parts of chloroform/methanol (2:1 v/v) [34], one part of chloroform and one of water. Chloroform phase containing lipid fraction was removed, dried under nitrogen stream and applied onto silica gel G precoated thin-layer chromatography plates. Phospholipids were separated by one-dimensional two-solvent system chromatography [35]. PC fraction was detected with iodine vapors, and the radioactivity incorporated was visualized by autoradiography and quantified in a liquid scintillation counter.

2.6. Analysis of choline-containing metabolites

Tissue slices (10–20 mg wet weight) were collected in 200 μL of ice-cold Tris–HCl buffer, pH 7.4, containing 5.5 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$ and 1 mM $CaCl_2$, and incubated at 37° with [methyl-^{3}H]choline in a metabolic shaking bath in an atmosphere of 95% O_2/5% CO_2, both in the absence and in the presence of SC-560 or NS-398. After 90 min, samples were rinsed with ice-cooled incubation medium and lipids were extracted by the modified Bligh and Dyer method described by Tran *et al*. [36]. Tissue was homogenized in methanol/HCl (100:1 v/v) and a monophasic extraction was first obtained with the addition of chloroform and ammonium acetate to the homogenate in order to achieve the ratio of 1:2:0.5 by volume of chloroform/methanol/0.1% ammonium acetate. Samples were then processed as previously described and choline-containing metabolites (phosphorylcholine and CDP-choline) obtained from the aqueous phase and resolved by thin-layer chromatography with a solvent system consisting of methanol/0.6% NaCl/NH_4OH (50:50:5 v/v/v). PC in the lower chloroformic phase was separated as described above. Radioactivity in each fraction was quantified by liquid scintillation counting.

2.7. Assay of phospholipid biosynthetic enzymes

CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) activities were assayed by the procedure described by Vance and Vance [37]. For CCT assays, microsomal or nuclei membranes (100–200 μg of protein) were incubated for 10 min with [methyl-^{14}C]phosphocholine (50 μCi/mol) and 12 mM CTP at 37° in a buffer containing 80 mM Tris–succinate, pH 7.4, and 48 mM magnesium acetate in a total volume of 100 μL. The reaction was terminated by immersion of the reaction tube in boiling water for 2 min. Radioactive CDP-choline was separated by thin-layer chromatography by using methanol/0.6% NaCl/NH_4OH (50:50:5 v/v/v) as a solvent system, and quantified by liquid scintillation counting. CPT assay was carried out in 100 μL of a mixture containing 50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 1.6 mM diacylglycerol and 10–30 μg of microsomal protein. The reaction was initiated by the addition of 3 μL of [methyl-^{14}C]CDP-choline (312 μCi/mmol), developed for 5 min at 37°, and terminated by adding 2 mL of chloroform/methanol (1:2 v/v). The ^{14}C-product was first extracted in the chloroformic phase, then transferred to scintillation vials, dried under a nitrogen stream, and finally 6.0 mL of toluene/omnifluor (0.4%) were added. Radioactivity was quantified in a liquid scintillation counter. Protein concentration was estimated by the method of Lowry *et al.* [38].

2.8. Statistic analysis

The results were expressed as the mean ± SEM. Data from controls and different treatments were analyzed by ANOVA and significant differences were assessed by "*a posteriori*" Scheffé test ($P < 0.05$). The correlation studies between phosphatidylcholine and prostaglandin synthesis were analyzed by applying lineal regression ($P < 0.05$, $r^2 > 0.84$).

3. Results

3.1. Rat renal papillary PC biosynthesis is modulated by endogenous COX-2 activity

Previous works from our laboratory showed that indomethacin, a non-specific COX inhibitor, causes a concentration-dependent inhibition on PC biosynthesis which is reverted by exogenous added prostaglandins [19]. In order to evaluate if phospholipid turnover depends on COX-1- or COX-2-mediated prostaglandin synthesis, we studied the incorporation of [^{3}H-methyl]choline ([^{3}H]choline) or [^{32}P]orthophosphate (^{32}Pi) both in the absence and in the presence of different concentrations (10^{-8} to 10^{-4} M) of SC-560 and NS-398, COX-1 and COX-2 specific inhibitors [28], respectively (Fig. 1). The incorporation of radioactive precursors to PC, which is indicative of PC biosynthesis, is considered a parameter of PC turnover at the steady-state equilibrium, since no changes are observed in PC mass content during the incubation period [12].

Neither [^{3}H]choline nor ^{32}Pi incorporation to PC was modified by COX-1 inhibitor SC-560 up to a concentration of 10^{-4} M, when it is considered to lose its selectivity (Fig. 1A and B). By contrast, COX-2 inhibitor NS-398, affected the incorporation of both radiolabeled precursors. Low concentrations (10^{-8} and 10^{-7} M) of NS-398 enhanced ^{3}H-PC and ^{32}P-PC production reflected by an increase in [^{3}H]choline and ^{32}Pi incorporation to PC of 28 and 64%, and 48 and 37%, at 10^{-8} and 10^{-7} M, respectively (Fig. 1C and D). Higher concentrations of NS-398 caused a concentration-dependent decrease in the incorporation of both [^{3}H]choline as well as ^{32}Pi to PC (Fig. 1C and D). No changes in PC mass were observed during the incubation time period. These results suggest that COX-2 activity, but not COX-1, is implicated in the regulation of PC biosynthesis.

3.2. Prostaglandin D_2 (PGD_2) synthesis depends on nuclear-cyclooxygenase-2 (COX-2)

With the purpose of establishing the type of prostaglandin affected by COX-2 inhibition, the biosynthesis of individual prostaglandins, both in the absence and in the presence of different concentrations of SC-560, NS-398 or indomethacin was studied. Fig. 2A–C shows the concentration-dependent inhibitory curves for PGE_2, $PGF_{2\alpha}$ and PGD_2 synthesis, respectively. COX-1 as well as COX-2 seem to be involved in PGE_2 synthesis, since both inhibitors, SC-560 and NS-398 blocked PGE_2 synthesis in a concentration-dependent manner (Fig. 2A). However, while COX-1 inhibitor blocked PGE_2 from 10^{-7} M, COX-2 inhibition was apparent from 10^{-5} M, demonstrating that PGE_2 synthesis is more sensitive to COX-1 than to COX-2 inhibition. $PGF_{2\alpha}$ synthesis was blocked by COX-1 specific inhibitor SC-560 and indomethacin but was refractory to the action of NS-398 (Fig. 2B). By contrast, PGD_2 synthesis was completely refractory to COX-1 inhibition while COX-2 specific inhibitor NS-398 and indomethacin caused a biphasic concentration–response curve. Low concentrations of inhibitors increased PGD_2 synthesis, showing the highest stimulatory effect between 10^{-8} and 10^{-7} M. Between 10^{-7} and 10^{-6} M, the inhibitory effect of the drugs started, which was reflected by a sharp descendent slope, reaching 75% of inhibition of PGD_2 production at 10^{-4} M of NS-398 and indomethacin (Fig. 2C).

It has been reported that both isoforms, COX-1 and COX-2, can be associated to both the endoplasmic reticulum and the nuclear envelope membranes [20]. Therefore, COX activity in enriched-nuclei fraction and in microsomes was studied (Fig. 3). Fig. 3A and B shows PGE_2, $PGF_{2\alpha}$ and PGD_2 synthesis as a function of the incubation

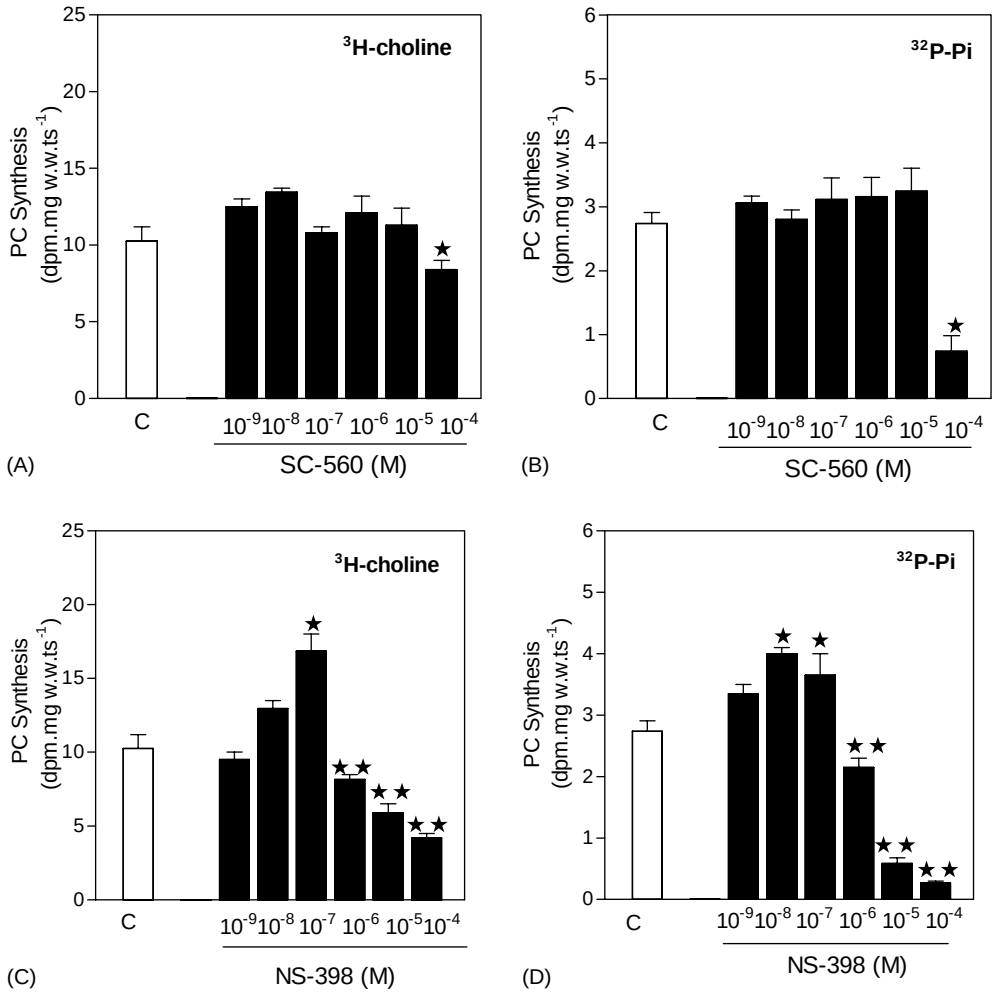

Fig. 1. Effect of COX-1 and COX-2 specific inhibitors on [methyl-^{3}H]choline or [^{32}P]-Pi incorporation into PC. Papillary samples (5 mg) were collected in a Tris–HCl solution, pH 7.4, containing electrolytes and 5.5 mM glucose and incubated up to the steady-state equilibrium both in the absence and in the presence of different concentrations of SC-560 (A and B) and NS-398 (C and D). Incubations were stopped by adding chloroform/methanol (2:1) and phospholipids extracted and separated by TLC as described in Section 2. Results expressed as dpm 10^{-3}/mg w.w.ts, represent the mean ± SEM of three individual experiments.

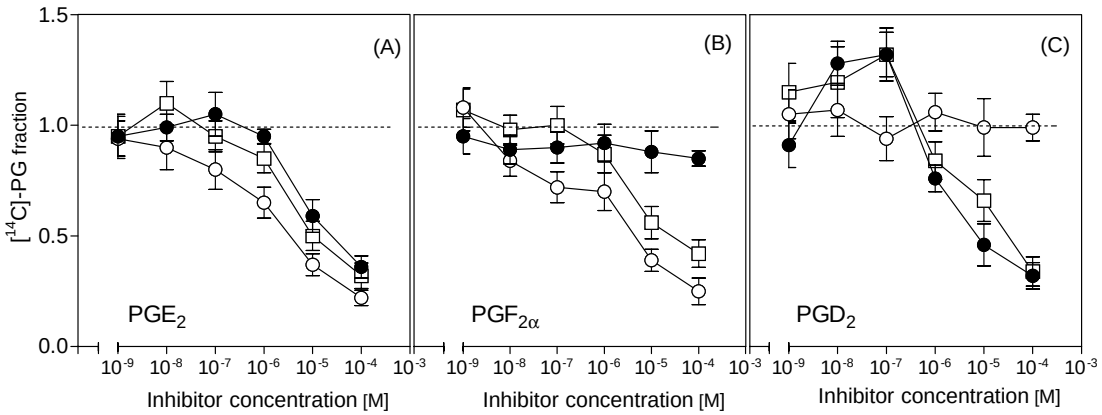

Fig. 2. Effect of COX-1 and COX-2 inhibitors on prostaglandin biosynthesis. Papillary homogenates (100 μg of protein) were incubated for 30 min with 30 μM [^{14}C]arachidonic acid without shaking or any other stimulus, both in the absence and in the presence of different concentrations of SC-560 (○), NS-398 (●) and indomethacin (□). Radiolabeled prostaglandins were extracted from the incubation media, separated by TLC and quantified as described in Section 2. The results are expressed as the ratio of treatment respect to control values. Each point represents the mean ± SEM of three determinations. Significantly different from control value with ★$P < 0.05$.

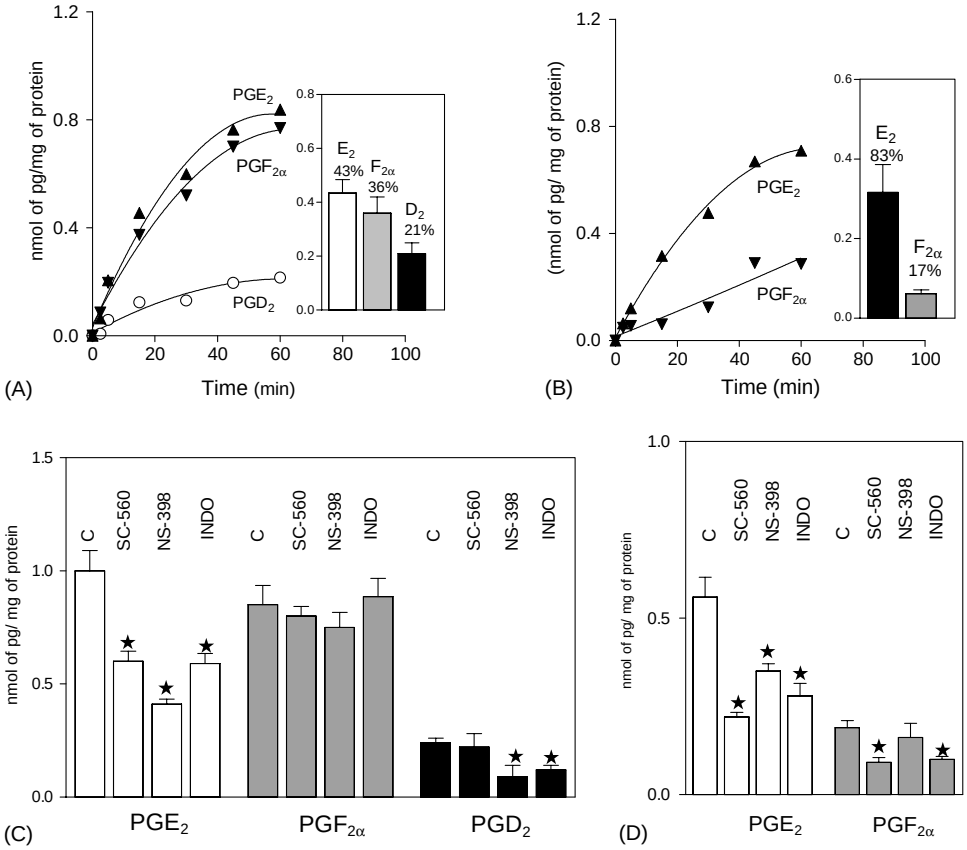

Fig. 3. Prostaglandin biosynthesis in subcellular compartments: effect of COX-1 and COX-2 inhibitors. Aliquots of pure nuclear (A) and microsomal (B) fractions (150 μg of protein) were incubated in the presence of 30 μM [^{14}C]arachidonic acid without shaking or any other stimulus, for different time periods. The prostaglandin profiles (insets) were obtained at 10 min incubations (in microsomal fraction, PGD_2 biosynthesis was not detected). In another set of experiments, nuclei (C) and microsomes (D) aliquots were incubated both in the absence and in the presence of SC-560, NS-398 and indomethacin at IC_{50} concentrations. Radiolabeled prostaglandins were extracted from the incubation media, separated by TLC and quantified as described in Section 2. Each point represents the mean ± SEM of three determinations. Significantly different from control value with ★$P < 0.05$.

time in both microsomal and nuclear fractions. Nuclear fraction was able to form PGE_2, $PGF_{2\alpha}$ and PGD_2 representing 43, 36, and 21% of total prostaglandin synthesis, respectively (Fig. 3A). In contrast, microsomal fraction generated PGE_2 (83% of microsomal total prostaglandin synthesis) and $PGF_{2\alpha}$ (17%) while no PGD_2 production was detected (Fig. 3B). Fig. 3C and D shows the effect of inhibitors on individual prostaglandin synthesis at a concentration that caused 50% inhibition of total prostaglandin synthesis (data not shown). Nuclear synthesis of PGE_2 was blocked by the three drugs assayed but was more sensitive to NS-398 (60% decrease) than to SC-560 and indomethacin (39 and 40% decrease, respectively). Nuclear $PGF_{2\alpha}$ production was not affected by any of the inhibitors used. PGD_2 synthesis was significantly blocked by NS-398 and indomethacin (63 and 50% decrease, respectively) but was refractory to SC-560 (Fig. 3C). Microsomal PGE_2 synthesis fell to 39, 63 and 50% in the presence of SC-560, NS-398 and indomethacin, respectively. Synthesis of $PGF_{2\alpha}$ associated to microsomes was affected by SC-560 and indomethacin (52 and 48% decrease, respectively) but was not significantly affected by the COX-2 inhibitor (Fig. 3D). All together, the above results evidenced that basal papillary prostaglandin synthesis is dependent on COX-1 as well as on COX-2 activities. However, specific isoform and subcellular compartmentalization are needed for the synthesis of individual prostaglandins. This fact is right evident in the case of PGD_2, which seems to be exclusively synthesized in nuclear fraction by COX-2 activity.

3.3. Basal rat renal papillary PC synthesis is dependent on COX-2-mediated PGD_2 biosynthesis

In order to obtain complementary data about the relationship between PC maintenance and papillary COX-2-mediated PGs synthesis, we established the correlation degree between both processes in the presence of NS-398. Fig. 4 shows the NS-398 concentration–response curves for PC and PG synthesis and the correlation factors r^2 calculated by lineal regression (bottom of each graph). The correlation degree between PC and PG synthesis was highly statistically significant all over the range of concentrations of the inhibitors used only in the case of PGD_2 (r^2: 0.9506 and 0.9211, ^{3}H-PC and ^{32}P-PC, respectively) (Fig. 4A). PGE_2 showed no correlation at low inhibitor concentrations (between 10^{-9} and 10^{-7} M) but higher

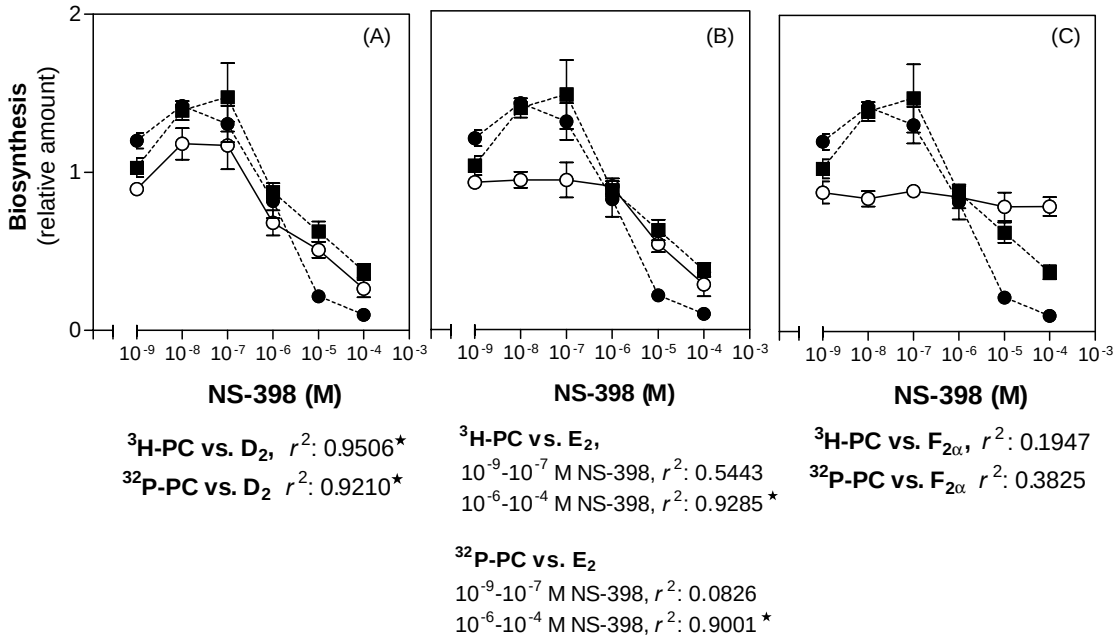

Fig. 4. Relationship between PC and papillary PG synthesis in the presence of COX inhibitors. The r^2 values between the PC biosynthesis decrease and PGs decrease in the presence of different concentrations of COX inhibitors were calculated by applying lineal regression statistical method. In each panel: (○), PG decrease; (●), ^{3}H-PC; (■), ^{32}P-PC.

NS-398 concentrations (10^{-6} to 10^{-4} M) showed high correlation degree (Fig. 4B) with both radioactive precursors. No correlation at all was observed between PC synthesis and $PGF_{2\alpha}$ production (Fig. 4C).

3.4. Nuclear CCT and CPT are targets for the COX-2 generated PGD_2 modulation of papillary PC biosynthesis

In mammalian kidney, PC synthesis occurs by the CDP-choline pathway described by Kennedy [44]. This metabolic route begins with the formation of choline-P (P-chol) by the catalytic action of choline kinase enzyme; choline-P is then converted into CDP-choline by CTP:phosphocholine cytidylyltransferase and, in the last step, choline:1,2-diacylglycerol cholinephosphotransferase transfers a phosphorylcholine group from CDP-choline to diacylglycerol, thus forming new PC moieties [10]. In order to approach the molecular level at which COX-2 inhibition causes PC synthesis impairment, we next studied the labeling of choline-containing metabolites of the CDP-choline pathway after NS-398 treatment (Table 1). This protocol allows an evaluation of the PC biosynthetic machinery preserving cell structure and physiology. As seen in Table 1, from the total amount of radioactive free-choline present in the aqueous phase, 43% was converted into P-chol and 26% into CDP-chol in control experiments. In the same experiments, the radiolabeled PC represented 0.14%. Pre-treatment with 2.5 μM NS-398 (concentration that causes 50% decrease in ^{3}H-PC synthesis, Fig. 2C) did not considerably change either free-choline or P-chol content, but a significant drop of CDP-chol was observed. These data, together with the fall in radioactive PC (Fig. 1C), show that due to COX-2 inhibition, PC synthesis impairment occurs at final stages of PC biosynthetic pathway, at the level of CDP-choline and PC formation.

We further evaluated CCT and CPT activities in isolated fractions. In a previous work, we have demonstrated that in papillary tissue PC synthesis is dependent on microsomal- and nuclear-associated enzyme activities [19].

Table 1
Effect of COX-2 inhibition on choline-containing metabolites

Treatment	Free-choline	P-choline		CDP-choline	
	pmol	pmol	P-chol/chol	pmol	CDP-chol/chol
None	61.16 ± 2.02	26.28 ± 2.31	0.420 ± 0.038	15.17 ± 0.89	0.258 ± 0.002
+NS-398	62.61 ± 2.20	23.60 ± 1.17	0.376 ± 0.017	12.74 ± 0.73*	0.197 ± 0.004*

Papillary slices were incubated with [^{3}H-methyl]choline both in the absence and in the presence of 2.5 μM NS-398. After incubation, samples were processed as described in Section 2 and soluble choline-containing metabolites were separated and quantified. Each value represent the mean ± SEM of three individual experiments.

* Significantly different from control values with $P < 0.05$.

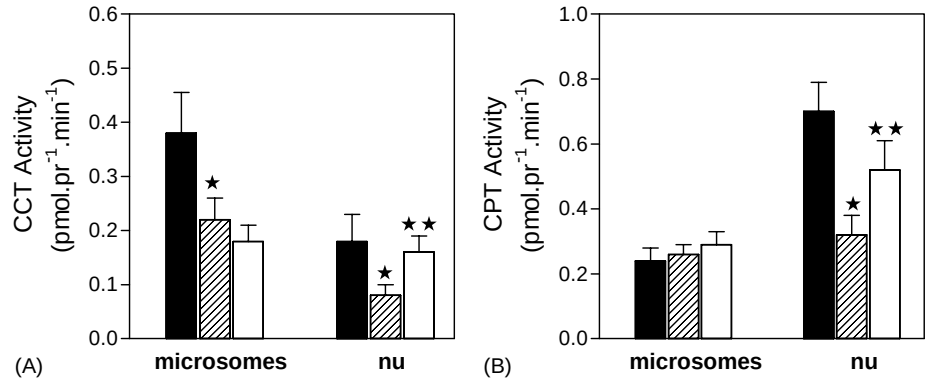

Fig. 5. Effect of NS-398 and PGD_2 on CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase activities. Renal papillary enriched-nuclear and microsomal fractions were obtained from control (*black bars*) and treated tissue (NS-398: *hatched bars*; NS-398 plus PGD_2: *white bars*) and incubated in the presence of the corresponding substrates and cofactors as described in Section 2. Enzyme activity was expressed as pmol product μg protein^{-1} ×10 min^{-1}. Significantly different from basal values with ★$P < 0.05$.

Therefore, we studied CCT and CPT activities in microsomal and enriched-nuclei fractions after pre-treatment of papillary samples with 2.5 μM NS-398 or 2.5 μM NS-398 plus 0.1 μM PGD_2. These results are shown in Fig. 5. NS-398 pre-treatment reduced microsomal as well as nuclear-associated CCT activities by a 43 and 56%, respectively (Fig. 5A). Nuclear associated CCT activity inhibition was reverted by PGD_2, while no change was found in microsomal enzyme activity. NS-398 treatment did not affect microsomal CPT, however, it decreased nuclear CPT activity by 54%, which was restored by PGD_2 addition (Fig. 5B). No direct effect was observed when NS-398 and/or PGD_2 were added during the assays performed on fractions from untreated-tissue (not shown). Indeed, Fig. 5 clearly indicates that, nuclear, but not microsomal, CCT and CPT activities are the targets for COX-2–PGD_2 dependent maintenance of PC biosynthesis.

4. Discussion

The purpose of the present work was to study which COX isoform is responsible for the prostaglandin production involved in the regulation of rat renal papillary phosphatidylcholine biosynthesis.

First, the present results demonstrate that COX-2 activity, but not COX-1, maintains PC biosynthesis in renal papillary cell membranes under basal conditions (Figs. 1, 4 and 5; Table 1). Since no physiological functions have been previously attributed to the constitutive expression of COX-2, the present results constitute the first demonstration of a physiological role for the constitutively expressed COX-2 in renal papillary tissue. Interestingly, low concentration of inhibitor increases PC biosynthesis, thus ensuring its renewal, while blockage is observed at higher concentrations of the drug. Consequently, we can speculate that COX-2 inhibitors could induce papillary damage so they are potentially risky drugs when used at high but not at low doses. This point can constitute a new aspect to take into account at the moment of using specific COX-2 inhibitors as pharmacological agents since they could affect papillary cell membrane homeostasis. We suggest that the maintenance of COX-2-mediated PC biosynthesis could be one of the biochemical mechanisms that explain the cytoprotective effect of COX-2 reported in collecting duct cells [15,25].

The second piece of evidence reported herein clearly demonstrates that COX-2-mediated PC biosynthesis is tethered to PGD_2 production at any concentrations of the inhibitor used (Fig. 4A). Thus, the dual effect on COX-2-mediated PGD_2 biosynthesis, is closely accompanied by the PC biosynthetic process (Fig. 4A). We have no explanation for the increase of PGD_2 synthesis exerted by low concentrations of COX-2 inhibitors, but increase in prostaglandin synthesis at low indomethacin concentrations was also reported by others in renal cortex [39], and a similar effect was also observed with COX-2 but not with COX-1 inhibitors in cultured fibroblasts as well as in COS cells [40]. However, no mechanism of action was reported in any case [41].

At high concentration (10^{-4} M), COX-1 inhibitor also affects PC synthesis, but does not seem to be PGD_2 mediated, since no decrease in PGD_2 was observed at 10^{-4} M SC-560 (Fig. 2C). Thus, the fall in PC biosynthesis observed at such condition has to be related to COX-1-mediated PGE_2 production (Figs. 2A and 3C). PGE_2 inhibition was also observed at low COX-1 inhibitor concentration (Fig. 2A), however it did not cause inhibition of PC synthesis (Figs. 1A, B and 4B). Taking together these results, we can suggest that renal papillary PC biosynthesis is indeed maintained by endogenous PGD_2 but probably, a low threshold amount of endogenous PGE_2 is also needed. By contrast, papillary $PGF_{2\alpha}$ does not exert any regulatory effect since no correlation at all exists between $PGF_{2\alpha}$ and PC biosynthesis (Fig. 4C).

It is interesting to note that, the biosynthesis of prostaglandins as well as the biosynthesis of PC seem to be

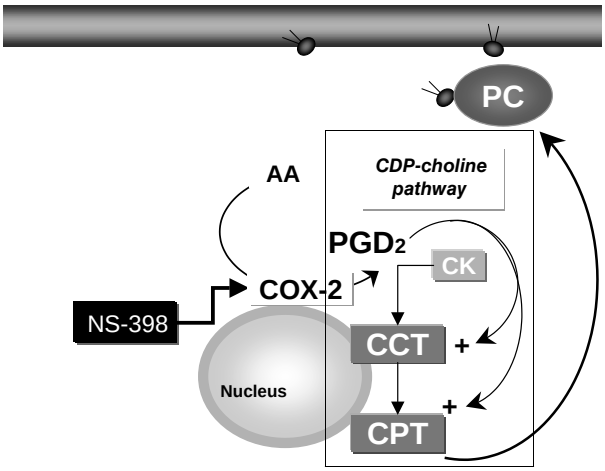

Fig. 6. Hypothetical representation of PC biosynthesis regulation by COX-2-dependent PGD_2 synthesis in rat renal papillary cell membranes.

highly compartmentalized processes in rat renal papillary cells. Apart from the fact that the regulation of PC synthesis occurs by the action of a specific prostaglandin, D_2, in a particular cellular compartment, the nucleus, generated by a specific COX isoform, COX-2, we have to add that such effect occurs by the maintenance of the basal activity of the two last CDP-choline pathway enzymes, CCT and CPT, located in the nuclear compartment.

In summary, the present work shows that COX-2 activity maintains PC biosynthesis in rat renal papilla. Such effect is mediated by a product of COX-2, PGD_2 (Fig. 6). Considering the importance of the preservation of cell membrane homeostasis for cell viability, the involvement of COX-2 in such process could not only be one of the mechanisms by which this enzyme acts as a ''survival protein'' but could also possess high pharmacological relevance. Thus, inhibitors of prostaglandins biosynthesis are commonly used as pharmacological treatment in medicine and papillary necrosis constitutes one of its iatrogenic consequences [42]. It has been reported that such an adverse effect is due to the fall in the prostaglandins involved in renal function regulation as a consequence of COX-1 but not of COX-2 inhibition [43]. However, the fact that COX-2 directly mediates in renal papillary PC resynthesis, herein demonstrated, could transform COX-2 inhibitors in drugs of higher risk than COX-1 inhibitors in evoking papillary necrosis. On the other hand, PGE_2 biosynthesis, mostly involved in Na^+ and water excretion, is inhibited by both inhibitors, which can be drugs of equal risk for the alteration of renal physiology.

The compartmentalized study of renal papillary prostaglandin synthesis and inhibition herein reported led us to distinguish the prostaglandin target of each COX inhibitor and consequently to dissect the potential deleterious side effects that each drug can produce in renal function. In conclusion, we suggest that non-steroidal anti-inflammatory drugs (NSAIDs) are drugs of risk for the kidney not only because they affect renal excretory function but also because of their deleterious effect on cellular membrane homeostasis.

Acknowledgments

This work has been supported by grants from the University of Buenos Aires, CONICET and Antorchas Foundation.

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