Biochemical and Biophysical Research Communications **277,** 599–603 (2000)
doi:10.1006/bbrc.2000.3725, available online at http://www.idealibrary.com on IDEAL®

Selective Inhibition of Cyclooxygenase-2 by C-Phycocyanin, a Biliprotein from *Spirulina platensis*

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Received September 23, 2000

We report data from two related assay systems (isolated enzyme assays and whole blood assays) that C-phycocyanin a biliprotein from *Spirulina platensis* is a selective inhibitor of cyclooxygenase-2 (COX-2) with a very low IC_{50} COX-2/IC_{50} COX-1 ratio (0.04). The extent of inhibition depends on the period of preincubation of phycocyanin with COX-2, but without any effect on the period of preincubation with COX-1. The IC_{50} value obtained for the inhibition of COX-2 by phycocyanin is much lower (180 nM) as compared to those of celecoxib (255 nM) and rofecoxib (401 nM), the well-known selective COX-2 inhibitors. In the human whole blood assay, phycocyanin very efficiently inhibited COX-2 with an IC_{50} value of 80 nM. Reduced phycocyanin and phycocyanobilin, the chromophore of phycocyanin are poor inhibitors of COX-2 without COX-2 selectivity. This suggests that apoprotein in phycocyanin plays a key role in the selective inhibition of COX-2. The present study points out that the hepatoprotective, anti-inflammatory, and anti-arthritic properties of phycocyanin reported in the literature may be due, in part, to its selective COX-2 inhibitory property, although its ability to efficiently scavenge free radicals and effectively inhibit lipid peroxidation may also be involved.

Key Words: **nonsteroidal anti-inflammatory drugs; phycocyanin; reduced phycocyanin; phycocyanobilin; cyclooxygenase-1; cyclooxygenase-2; inhibition.**

Cyclooxygenase (COX, Prostaglandin H Synthase) is a bifunctional enzyme catalyzing the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (1). Studies carried out so far suggest that there is a link between cancer and prostaglandins and it has been observed that tumor tissues contain higher levels of PGs (2). It is now known that at least two forms of cyclooxygenase enzyme exist (3). One of these forms, cyclooxygenase-1 (COX-1), is considered a constitutive form and is responsible for maintaining normal physiologic function and the PGs produced by this enzyme play a protective role. The other known form of the enzyme, cyclooxygenase-2 (COX-2), is an inducible form and its expression is affected by various stimuli such as mitogens, oncogenes, tumor promoters, and growth factors (4). COX-2 is the principal isoform that participates in inflammation, and induction of COX-2 is responsible for the production of PGs at the site of inflammation (4). It was shown that COX-2, and not COX-1, activity increases in malignant tissue from colorectal cancer, human gastric and breast tumors (5).

It is known that nonsteroidal anti-inflammatory drugs (NSAIDs) are effective against inflammation and are shown to inhibit PG biosynthesis which are the inflammatory mediators (6). However, the deleterious side effects of NSAIDs are that while they inhibit PG biosynthesis at inflammatory sites, they are also known to inhibit constitutive biosynthesis of PGs through the mediation of COX-1 (5, 7). So the adverse effects of currently available NSAIDs have limited their clinical usefulness. Hence, selective inhibition of COX-2 would prevent the formation of inflammatory PGs without affecting the COX-1 activity and, in fact, there is a concerted effort by various investigators to develop such inhibitors for therapeutic uses.

One of the process involved in carbon tetrachloride (CCl_4)-induced hepatotoxicity is the free radical-catalyzed lipid peroxidation or oxidative injury (8). It is known that lipid peroxidation mediated by free radicals activate cyclooxygenases resulting in the formation of PGs from arachidonic acid (9). Nonenzymatic free radical catalyzed and enzymatic cyclooxygenase mediated oxidation of arachidonic acid have been shown to be involved during CCl_4-induced hepatotox-

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0006-291X/00 $35.00

icity (9). Earlier studies have indicated that oxidative injury is the initial and cyclooxygenase-mediated inflammatory response is the secondary effect of CCl_4-induced hepatotoxicity (9). Recent studies have also demonstrated that one of the end products of lipid peroxidation viz. 4-hydroxy-2-nonenal, a breakdown product of hydroperoxy fatty acid, is a specific inducer of COX-2 expression (10). This suggests that during CCl_4-induced hepatotoxicity the level of COX-2 gets elevated.

C-Phycocyanin is one of the major biliproteins of *Spirulina platensis*, a blue-green algae. This water soluble protein pigment has significant antioxidant and radical scavenging properties (11–13). Earlier we demonstrated that phycocyanin significantly reduces R-(+)-pulegone (a potent hepatotoxin) and CCl_4-induced hepatotoxicity in rats (14). It effectively inhibited CCl_4-induced lipid peroxidation in rat liver *in vivo* (13). Recently it was demonstrated that oral administration of phycocyanin exerted anti-inflammatory effects in arthritis induced by zymosan in mice (15). It was suggested that the anti-inflammatory activity of phycocyanin could be due to its ability to inhibit arachidonic acid metabolism and to scavenge oxygen free radicals (15, 16). In fact, oxygen free radicals are believed to be involved in rheumatoid arthritis (17) and inhibitors of arachidonic acid metabolism are commonly used in the treatment of arthritis (18). However, the mechanism of action of phycocyanin is not clearly understood. The present study reports for the first time that phycocyanin is a selective COX-2 inhibitor with very low COX-2/COX-1 ratio and potency comparable to those of rofecoxib and celecoxib, the known selective COX-2 inhibitors.

MATERIALS AND METHODS

Chemicals. Arachidonic acid, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) was purchased from Sigma Chemical Co. (St. Louis, MO). Indomethacin was supplied by Cayman Chemical Co. (Ann Arbor, MI). PGE_2 EIA kit was from Assay Designs, Inc (Ann Arbor, MI). Celecoxib and rofecoxib were a generous gift from Unichem Laboratories (Mumbai, India). Recombinant human COX-2 was a generous gift from Shozo Yamamoto (School of Medicine, University of Tokushima, Japan).

C-Phycocyanin was isolated from *Spirulina platensis* and purified as reported earlier (19). The fractions showing absorbance ratio of 618 nm/280 nm greater than 4 were pooled and used in the present study. The purity and molecular mass of the purified phycocyanin were determined as reported earlier (13). The chromophore (phycocyanobilin) covalently attached to phycocyanin was reduced using solid $NaBH_4$ (13, 20). The phycocyanobilin (PCB) was cleaved from pure freeze-dried phycocyanin by alcoholysis (21). The PCB obtained was redissolved in chloroform containing 5% methanol, washed several times with water to remove impurities and evaporated under vacuum to dryness. The purity of phycocyanobilin was examined by HPLC analysis, using ODS reverse phase column with acetone: water (2.5:1.5, v/v) containing 2 mM H_3PO_4 and 0.5 mM NaH_2PO_4 as a solvent system (1.5 ml/min) and the column elute was monitored at 365 nm. The UV-visible spectra of PCB was recorded between 280–800 nm in methanol/2% HCl and the concentration was estimated at 374 nm using extinction coefficient of 47,900 $M^{-1}cm^{-1}$ (22).

Preparation of COX-1 and COX-2. Microsomal fraction of ram seminal vesicles containing cyclooxygenase-1 (COX-1) activity was prepared and the enzyme was purified as described earlier (23, 24). Briefly, the ram seminal vesicles were minced into small pieces and homogenized in the Tris–HCl buffer (100 mM, pH 8.0) containing EDTA (5 mM), and Diethyldithiocarbomate (DDC, 5 mM). The homogenate was subjected to differential centrifugation to obtain microsomal pellet. The microsomal pellet (105,000*g* pellet) was suspended in Tris–HCl buffer (25 mM, pH 8.0) containing EDTA (1 mM) and Triton X-100 (1%) and then centrifuged at 105,000*g* for 1 h at 4°C. The supernatant was subjected to DEAE-cellulose column chromatography and the fractions containing COX-1 activity were pooled and used as the source of enzyme.

Recombinant human cyclooxygenase-2 (COX-2) was expressed in *Spodoptera frugiperda* (Sf9) cells infected with bacculovirus containing human COX-2. After 72 h of infection, the cells were collected by centrifugation at 5000 rpm for 5 min. The pellet was suspended in Tris–HCl buffer (pH 7.2, 50 mM) containing EDTA (5 mM), sucrose (300 mM), DDC (5 mM), pepstatin (1 μg/ml) and phenol (1 mM) and sonicated for 3 min. The cell lysate was subjected to centrifugation (100,000*g* for 1 h) at 4°C and the microsomal pellet obtained was suspended in Tris–HCl buffer (pH 7.2, 25 mM) containing glycerol (0.5%), Tween 20 (0.8%) and phenol (1 mM). Protein was determined by the method of Bradford (25).

Spectrophotometric assay for COX-1 and COX-2. Enzymatic activity of COX-1 and COX-2 were measured as described earlier with little modification (26) using a chromogenic assay based on the oxidation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris–HCl buffer (pH 8.0, 100 mM), hematin (15 μM), EDTA (3 μM), enzyme (COX-1 OR COX-2, 100 μg) and test compound, phycocyanin (for COX-1, 1–100 μM in 12 μl of buffer, for COX-2, 0.03–30 μM in 12 μl of buffer). The mixture was preincubated at 25°C for 15 min. and then the reaction was initiated by the addition of arachidonic acid (100 μM in 5 μl of ethanol) and TMPD (120 μM in 3 μl of ethanol) in a total volume of 1.0 ml. The enzyme activity was measured by estimating the initial velocity of TMPD oxidation for the first 25 s of the reaction following the increase in absorbance at 603 nm. A low rate of nonenzymatic oxidation observed in the absence of COX-1 and COX-2 was subtracted from the experimental value while calculating the percent inhibition. The effect of different concentrations of indomethacin, celecoxib and rofecoxib (known inhibitors of COX-1 and COX-2) were examined under the same experimental conditions.

Human whole blood assay for COX-2. The assay was carried out as previously described (27). Briefly, freshly heparinized human whole blood was incubated with lipopolysaccharide (LPS) from *Escherichia coli* (100 μg/ml) and indicated concentration of test compound [0.01–3.0 μM, phycocyanin in buffer (5 μl); celecoxib in DMSO (5 μl)] for 24 h at 37°C. The level of PGE_2 formed was measured using EIA kit as per the protocol given by the company (Assay Designs, Inc., USA).

RESULTS

Inhibition of COX-1 and COX-2 by phycocyanin was analyzed in both cell-free and whole blood assay systems. The partially purified enzyme from ram seminal vesicles served as the source of COX-1, while the human recombinant enzyme formed the source of COX-2. The inhibition of COX-1 by phycocyanin, celecoxib, and indomethacin is shown in Fig. 1. It was demonstrated that phycocyanin and celecoxib at a concentration of 100 μM inhibited COX-1 activity almost completely

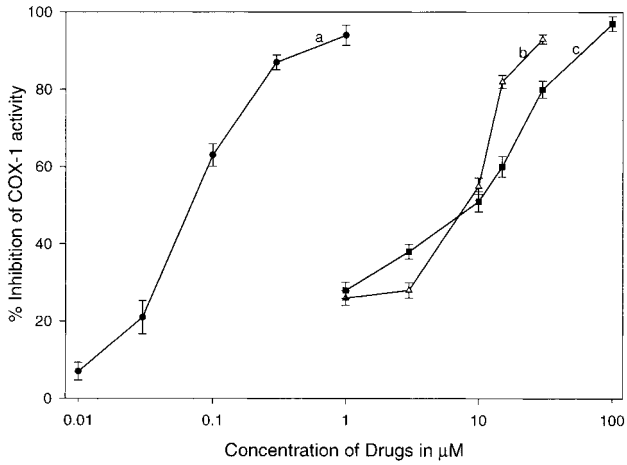

FIG. 1. The inhibitory effect of (a) indomethacin (0.01–1.0 μM), (b) phycocyanin (1.0–30.0 μM), and (c) celecoxib (1.0–100 μM) on ram seminal vesicle COX-1 activity. The results are expressed as % inhibition of COX-1 activity by inhibitors. The experimental details are as described under Materials and Methods.

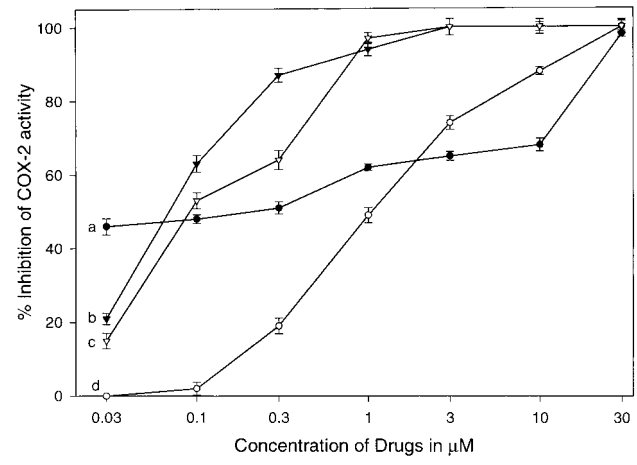

FIG. 2. The inhibitory effect of (a) phycocyanin, (b) rofecoxib, (c) celecoxib, and (d) indomethacin (0.03–30.0 μM) on human recombinant COX-2 activity. The results are expressed as % inhibition of COX-2 activity by inhibitors. The experimental details are as described under Materials and Methods.

(~95%), where as much lower concentration (~1 μM) of indomethacin was required to exhibit the same effect (Fig. 1). Indomethacin is the most potent inhibitor of COX-1 with IC_{50} value of 0.216 μM followed by phycocyanin (IC_{50}, 4.47 μM) and celecoxib (IC_{50}, 16.3 μM). The inhibition of COX-1 by phycocyanin was dose-dependent, but independent of the period of preincubation with the enzyme. Phycocyanin at a concentration of 1 μM inhibited COX-1 activity by 26% with or without preincubation of the enzyme with the inhibitor.

The present studies revealed that phycocyanin is a potent inhibitor of human recombinant COX-2 with an IC_{50} value of 180 nM (Table 1) which is much lower than the values obtained for celecoxib (255 nM) and rofecoxib (401 nM), the known selective inhibitors of COX-2. The extent of inhibition of COX-2 by phycocyanin was dependent on the preincubation period of the enzyme with inhibitor before the initiation of reaction with arachidonic acid. It was noticed that at 0.3 μM concentration of the inhibitors, highest percentage of inhibition of COX-2, was recorded for phycocyanin (48%) as compared to rofecoxib (21%) and celecoxib (15%) (Fig. 2). Based on the IC_{50} values for COX-1 and COX-2, the relative ratios of IC_{50} COX-2/IC_{50} COX-1 were calculated and the data is presented in Table 1. The COX-2/COX-1 ratio obtained for phycocyanin (0.04) is comparable to the values obtained for well-known selective COX-2 inhibitors such as celecoxib (0.015) and rofecoxib (0.0013) (Table 1). However, these values are significantly different from those obtained for indomethacin (8.1), the selective COX-1inhibitor.

TABLE 1

Drugs	IC_{50} (μM) COX-1	COX-2	COX-2/COX-1
Phycocyanin	4.5	0.18	0.04
Reduced phycocyanin	5.6	9.7	1.73
Phycocyanobilin	9.9	39.0	3.93
Celecoxib	16.3	0.26	0.015
Rofecoxib	>300*	0.4	<0.0013
Indomethacin	0.22	1.74	7.9

Note. The IC_{50} values of phycocyanin, reduced phycocyanin, phycocyanobilin, celecoxib, rofecoxib, and indomethacin in ram seminal vesicle COX-1 and human recombinant COX-2 activity. *Maximum dissolved concentration.

COX-2 activity was also determined in the human whole blood stimulated by LPS and measuring the release of prostaglandin E_2 (PGE_2). Phycocyanin at a concentration of 1 μM inhibited the release of PGE_2 to a significant extent (nearly 80% inhibition) with an IC_{50} value 80 nM. The results (Fig. 3) also indicate that phycocyanin inhibits PGE_2 synthesis in a dose-dependent manner. The IC_{50} value determined for celecoxib in the whole blood assay was found to be 28 nM.

To understand the role of chromophore in the inhibition of COX-1 and COX-2 by phycocyanin, we studied these activities in the presence of reduced phycocyanin as well as phycocyanobilin, the chromophore of phycocyanin. It was observed that both these compounds significantly lost the selectivity towards COX-2 inhibition (Table 1). However, there was not much change in the inhibition of COX-1 by these two compounds and as a result the relative ratios of IC_{50} COX-2/IC_{50} COX-1 for reduced phycocyanin and phycocyanobilin increased by several folds as compared to native phycocyanin.

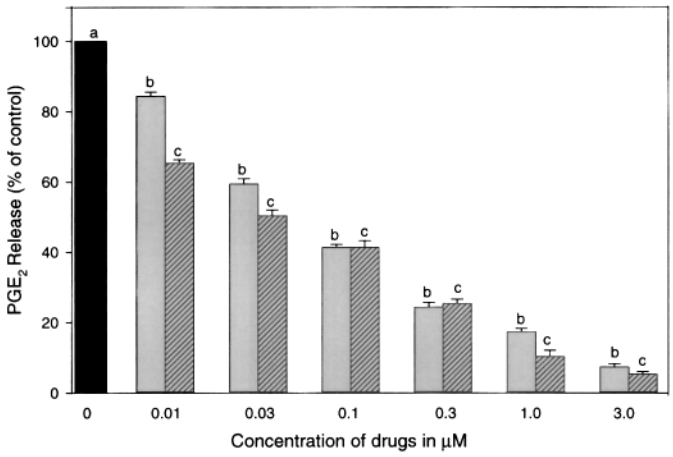

FIG. 3. Effect of celecoxib and phycocyanin on LPS-stimulated PGE_2 synthesis in human whole blood assay. PGE_2 levels determined without drug taken as the maximal PGE_2 synthesis (i.e., 100%) was used as control. (a) Control, (b) celecoxib, and (c) phycocyanin. The results are expressed as % of PGE_2 release to plasma (% of control). The experimental details are as described under Materials and Methods.

DISCUSSION

Cyclooxygenase, a key enzyme involved in the biosynthesis of prostaglandin (1), plays an important role in inflammation and variety of other disorders (2, 5, 9). With the discovery of inducible form of cyclooxygenase, COX-2 (3), it has been postulated that PGs that contribute to inflammatory process are derived exclusively from COX-2, while many of the "house-keeping" effects of COX appear to be mediated by COX-1. Selective inhibitors of COX-2 would exhibit anti-inflammatory and analgesic effects without the deleterious side effects of NSAIDs. Hence, a lot of interest is being shown to develop drugs that would selectively inhibit COX-2 without affecting COX-1 activity. These efforts have led to the introduction of selective COX-2 inhibitors such as celecoxib by Searle and rofecoxib by Merck, as new class of NSAIDs into the market.

In the present study it has been shown that phycocyanin is a selective inhibitor of COX-2. It is a more potent (IC_{50} 180 nM) inhibitor of COX-2 activity than celecoxib (IC_{50} 255 nM) and rofecoxib (IC_{50} 401 nM). The human whole blood COX-2 assay provides an additional and a more relevant measure of COX-2 inhibition selectivity under a pathophysiological environment rich in plasma protein and cells (27, 28). In the present study it was observed that phycocyanin very efficiently inhibited COX-2 activity with an IC_{50} value of 80 nM in the human whole blood assay, wherein the fresh heparinized human whole blood was incubated with LPS and the PGE_2 formed is measured. In fact the IC_{50} value obtained in the whole blood assay (80 nM) is much lower than the value obtained with the partially purified enzyme (180 nM). Inhibition of COX-2 activity is a favorable condition for treating inflammation, arthritis, and preventing cancer (29). Earlier studies have demonstrated the anti-inflammatory property of phycocyanin (15) and this property of phycocyanin can be explained, in part, by the specific inhibition of COX-2. The mechanism of inhibition of COX activity by phycocyanin appears to be similar to those reported for COX-2 selective inhibitors, occurring via a time-dependent mechanism leading to a possible formation of a tightly bound inhibitor complex (26, 30). Additionally, phycocyanin has been shown earlier to possess radical scavenging and antioxidant properties (11–13) which could also contribute substantially towards its anti-inflammatory and anti-arthritic effect. It is increasingly recognized that reactive oxygen species are involved in rheumatoid arthritis (17, 31). When compared to the toxicities associated with the currently available anti-inflammatory drugs and their activity as COX-2 inhibitors, phycocyanin will likely provide safer therapeutic alternatives since it is as efficacious as currently used drugs (NSAIDs), if not more. But most importantly, this water-soluble biliprotein is from a natural source and least toxic. Recently it has been shown that resveratrol, a phytoalexin found in grapes and other foods, inhibits COX-2 activity and suppresses the activation of COX-2 gene expression (32). It is also known that compounds such as radicicol, genistein, curcumin, and retinoids inhibit COX-2 gene expression (33).

Earlier, we demonstrated that phycocyanin significantly reduces R-(+)-pulegone and CCl_4-induced hepatotoxicity in rats (14). One of the process involved in CCl_4-induced hepatotoxicity is the free radical-catalyzed lipid peroxidation (8) which is accompanied by activation of cyclooxygenase and increased synthesis of PGs (9). The hepatoprotective effect of phycocyanin could be due to its ability to efficiently scavenge free radicals (13) and inhibit lipid peroxidation as well as COX-2 activity.

IC_{50} COX-2/IC_{50} COX-1 ratios provide a useful comparison of relative values for a series of NSAIDs tested in the same system. However, this ratio for a particular NSAID will vary according to whether it is measured using intact cells, cell homogenates, purified enzymes, or recombinant proteins expressed in bacterial, insect, or animal cells. It will also vary when measured in different types of cells derived from various species (34). Studies indicate that a high degree of *in vitro* biochemical selectivity for COX-2 will be required in order to achieve effective functional selectivity *in vivo.* The ratio demonstrates the relative selectivity of NSAIDs towards the two COX isoforms and low ratios indicate a preferential inhibition of COX-2. In the present study COX-2/COX-1 ratio of the IC_{50} values calculated for phycocyanin *in vitro* with the partially purified enzymes is very low and comparable to those calculated for celecoxib and rofecoxib.

It is known that the active site of COX-2 is larger than that of COX-1 so that it can accommodate bigger structures (35). Phycocyanin is significantly much bigger in size (~37.5 kDa) than NSAIDs. Its three-dimensional structure probably would facilitate the proper binding with the active site of COX-2. In fact, reduced phycocyanin where the C-10 methine bridge in the chromophore is reduced (22) is significantly a less active COX-2 inhibitor and also lost COX-2 selectivity (Table 1). It is quite possible that the reduced phycocyanin with an altered native conformation may not favor proper binding at the active site. This appears to be true with phycocyanobilin, the chromophore of phycocyanin, which also significantly lost COX-2 selectivity (Table 1) suggesting that the apoprotein plays a role in the inhibition of COX-2.

Preliminary studies indicated that phycocyanin induced apoptosis in RAW 264.7 macrophages in which COX-2 activity was stimulated by LPS. Some of the selective COX-2 inhibitors exhibit this property (36). It has also been observed that phycocyanin failed to inhibit human 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenase (unpublished data).

In summary, we have demonstrated that phycocyanin is a selective inhibitor of COX-2 activity with potency comparable to celecoxib and rofecoxib, the known selective COX-2 inhibitors. Reduced phycocyanin and phycocyanobilin are poor inhibitors of COX-2 activity without COX-2 specificity.

ACKNOWLEDGMENTS

Financial assistance from CSIR, DBT, DST (New Delhi, India), and JNCASR (Bangalore, India) is gratefully acknowledged.

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