Carcinogen substrate specificity of human COX-1 and COX-2

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The activation of carcinogenic aromatic and heterocyclic amines and benzo[a]pyrene-7,8-diol to intracellular electrophiles by prostaglandin H synthase (COX) is well documented for ovine sources of this enzyme. Here, the arachidonic acid-dependent activation of substrates by human (h)COX-1 and -2 is examined, utilizing recombinant enzymes. The COX-dependent activation of benzo[a]pyrene (BP), 4-aminobiphenyl, (+)-benzo[a]pyrene-7,8-diol, (+)-benzo[a]pyrene-7,8-diol, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx), 2-amino-3-methylimidazo[4,5-b]pyridine (PhIP), and 4,4’-methylenebis(2-chloroaniline) (MOCA) is assessed by means of COX-catalyzed, covalent DNA binding. The hCOX isozymes activated all substrates tested, activation varied from barely detectable for IQ (0.76 and 1.52 pmol bound/mg DNA for COX-1 and -2, respectively) to a high of 65 and 117 pmol bound/mg DNA for COX-1 and -2, respectively, for the activation of MOCA. BZ, which is an excellent peroxidase substrate, did not exhibit high DNA binding levels in hCOX assays and this phenomenon was found to be due to high levels of binding to protein, which effectively competed with the DNA for binding in the assay. The demonstrated ability of the COX enzymes to activate a variety of environmental and dietary carcinogens indicates a potential role for COX in the activation pathway of aromatic and heterocyclic amines and polycyclic hydrocarbons at extra-hepatic sites during early or late stages of carcinogenesis.

Introduction

Prostaglandin H synthase (COX/PGHS) is the enzyme responsible for catalysis of the committed step in prostanooid biosynthesis. There are two distinct isoforms of this enzyme, a constitutively expressed isoform (COX-1) and an inducible isoform (COX-2). COX-1 is expressed in virtually all extra-hepatic tissues, though not in all cell types within a tissue (1–3). Conversely, COX-2 is present constitutively in few tissues (brain, testis, kidney), but its expression can be induced by a variety of mediators, including cytokines, growth factors, tumor promoters (4–6), bile acids (7), xenobiotic response element inducers (8,9) and UVB irradiation (10). Overexpression of COX-2 is seen in several neoplastic tissues, e.g. colon (11,12), breast (13) and lung (14,15). The two COX isozymes are bifunctional enzymes (cyclooxygenase and hydroperoxidase catalytic sites) that share ~60% amino acid sequence homology. Fatty acid substrate specificities (16,17), intracellular localization (18) and hydroperoxide initiator requirements (19) for the two isoforms of COX have been investigated.

The ability of human COX (hCOX) to activate several carcinogenic compounds and possible differences in substrate specificity between hCOX-1 and hCOX-2 are examined here. For these studies, an arachidonic acid-dependent, COX-catalyzed DNA binding assay, as measured by the formation of stable covalent adducts to DNA, was utilized to assess the bioactivation of the test substrates by the enzymes. Although activation of carcinogens by COX has been reported, the majority of this work has been performed with either ovine sources of enzyme (oCOX) or with microsomal preparations of human tissue (20–23). Studies have been performed with recombinant human preparations for 2-amino-1-methylimidazo[4,5-f]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-b]pyridine (PhIP) (25) and 2-aminofluorene (26), but appreciable activation has only been detected with the latter substrate. For the substrate work performed herein, two transfected Chinese hamster ovary (CHO) cell lines, expressing full-length hCOX-1 or -2 (24), were utilized.

The compounds selected for these COX bioactivation studies are well known industrial chemicals or dietary components. A detailed review of these chemicals can be found elsewhere (27). Briefly, benzidine (BZ), a known human carcinogen, now usually considered only as a model peroxidase substrate, still has some occupational exposure. Moreover, BZ is often a contaminant of hundreds of BZ-based azo dyes, such as Direct Black 38 that is used in newspaper ink. 4-Aminobiphenyl (ABP), another known human occupational carcinogen, is also a ubiquitously occurring compound present in the environment with high levels found in cigarette smoke. 4,4’-Methylenebis(2-chloroaniline) (MOCA) is a curing agent widely used in the manufacture of polyurethane and is considered a probable human carcinogen. 2-Amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx), IQ and PhIP are compounds formed during the high-temperature cooking of meat and have been implicated in the etiology of human colon cancer. These compounds were considered potential substrates for the hydroperoxidase activity of hCOX, since oCOX is capable of oxidizing the amino group of such compounds (Figure 1).

Benzo[a]pyrene (BP), another probable human carcinogen, is a ubiquitous combustion product found in polluted air,
Fig. 1. Activation of substrates by COX enzymes. The cyclooxygenase and hydroperoxidase catalytic sites can both participate in the activation of substrates. Peroxy radicals are formed at the cyclooxygenase catalytic site that can introduce an oxygen across an isolated double bond, as in the activation of (+)-BP-7,8-diol. A variety of aromatic amines and phenols may function as a reducing substrate at the hydroperoxidase catalytic site. Shown are ABP and BZ.

cigarette smoke, soot, tar, oils and flame-cooked foods. The metabolism of BP to BP-7,8-diol and to its DNA-reactive BP-7,8-diol-9,10-oxide by oCOX-1 has been thoroughly investigated and reviewed by Marnett (28), and proceeds through the formation of peroxyl radicals by the cyclooxygenase activity of the enzyme (Figure 1). These cyclooxygenase-formed peroxyl radicals are capable of adding an oxygen molecule across the isolated C9–C10 double bond of BP-7,8-diol forming BP-7,8-diol-9,10-oxide. Of special interest concerning the activation of BP-7,8-diol is the fact that BP has been shown to induce the expression of COX-2 (8), so that BP and its metabolites function not only as inducers, but also as substrates for COX. In our work, the COX-catalyzed activation of (+)-BP-7,8-diol as well as (±) BP-7,8-diol is examined.

Materials and methods

Chemicals

All radioactive substrates were purchased from Chem-Syn Science Labs (Lenexa, KS). [1H] Substrates included: [2,2′-3H]BZ (sp. act. 26.4 mCi/mmol), [2,2′-3H]ABP (49.2 mCi/mmoll), [1,3-3H]-(+)BP-7,8-diol (36.9 mCi/mmoll), [1,3-3H]-(+)BP-7,8-diol (19.3 mCi/mmol) and [ring-3H] PhIP (85 mCi/mmoll). 1C-labeled substrates included: [2-14C]IQ (45.3 mCi/mmoll), [2-14C]MeloQ (13.8 mCi/mmoll) and [4,4′-methylene-14C]Moca (8.66 mCi/mmoll). The purity of substrates was verified by HPLC and, if necessary, re-purified with Waters Oasis HLB extraction cartridges (Waters, Milford, MA), according to manufacturer’s instructions, yielding purities of >95%.

All additional chemicals utilized (unless otherwise noted) were purchased from the Sigma Chemical Co. (St Louis, MO) and were of the highest purity available.

Cell culture

The CHO cells utilized as an enzyme source were a gift from Dr Stacia Kargman of the Merck Frosst Center for Therapeutic Research in Quebec, Canada. The construction and care of these cells has been documented elsewhere (24) and the published protocol was followed with minor adaptation. Briefly, the hCOX-1 expressing CHO line was grown and selected using G418 (Gibco BRL, Grand Island, NY) in HyQ-CCM5 medium (Hyclone Labs, Logan, UT) supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin. The hCOX-2 transfected CHO cells were selected using 25 µM 1-methionine sulfoximine in Glasgow’s MEM with l-glutamine (BioWhittaker, Walkerville, MD) and supplemented with 10% dialyzed fetal bovine serum, 100 µg/ml streptomycin, 100 U/ml penicillin, 1.0 mM sodium pyruvate, 60 µg/ml 1-asparagine, 7 µg/ml ribonucleosides and non-essential amino acids. Cells were grown as a monolayer culture at 37°C in 5% CO₂. Cells were harvested by removing the media on confluent cultures, rinsing once with Hank’s Balanced Salt Solution-EDTA, then covering with the same solution and incubating for 15 min. The cells were next transferred to 50 ml conical tubes and cell pellets were obtained by centrifugation (3300 g for 15 min at 4°C).

Enzyme preparation

Harvested cells were prepared according to published procedures (29). The cell pellets were allowed to swell in a hypotonic buffer and then lysed by passage through a bent 26-gauge needle. The cell lysate was then centrifuged at 3300 g for 15 min at 4°C. The supernatant was transferred to new tubes and centrifuged at 100 000 g for 1 h at 4°C. The resultant microsomal pellet was resuspended in a solution consisting of 0.25 M sucrose, 10 mM Tris–acetate buffer (pH 7.4) and 20% glycerol. Protein concentrations were determined by the biuret method (30).

Ovine COX standards were obtained from Oxford Biomedical Research, Oxford, MI (oCOX-1, product no. PG01) and from Cayman Chemical Company, Ann Arbor, MI (oCOX-2, product no. 60120).

Hydroperoxidase activity

Previous work has demonstrated that the catalytic activities of COX-1 and -2 are similar (31). Hydroperoxidase activity was thus analyzed to determine approximate amounts of hCOX enzyme present in the CHO microsomal fractions as well as levels of oCOX-1 and -2 in different preparations. This was determined utilizing a spectrophotometric assay for the hydroperoxidase-catalyzed oxidation of N,N′,N′-tetramethyl-p-phenylenediamine (TMPD; ref. 46) containing 100 µg of microsomal preparation. Double reciprocal plots generated utilizing H₂O₂ concentrations from 5–20 µM with recombiant hCOX-1 and -2 preparations were essentially identical (results not shown), demonstrating that approximately equal amounts of enzyme were expressed per microgram of microsomal protein. Additionally, comparison of line cell pellets derived from the double reciprocal plots of the hCOX isozymes and oCOX-1 allowed the estimation of hCOX activity in terms of units of oCOX-1 activity. For the H₂O₂-catalyzed oxidation of TMPD, a value of 0.5 U oCOX-1 activity/µg of hCOX microsomal protein was obtained.

Enzyme assays

The activation of substrates was determined by the arachidonic acid-dependent, DNA binding of radiolabeled substrates essentially as described previously (23). The assays consisted of 50 mM potassium phosphate buffer (pH 7.4), 100 µM arachidonic acid (Nu-Chem Prep, Elysian, MN), 1 µM hematin, 2.5 mg calf thymus DNA, 20 µM [3H]- or [14C]-labeled substrate and 500 µg CHO microsomal protein or 50 U oCOX (activities were determined by the manufacturer, one unit is the amount of enzyme capable of consuming 1 nmol of oxygen per minute at 37°C in the production of PGG2) in a 1 ml total reaction volume. Reactions were initiated by the addition of enzyme and incubated at 37°C for 10 min, and terminated by the addition of 2 vol ice-cold ethanol; phenol (99:1). Reactions without enzyme, or without arachidonic acid, were used as controls (the values obtained were comparable). After termination, the DNA was purified by multiple solvent extractions and precipitations (32); the levels of bound substrate were then determined by liquid scintillation counting. The initial experiments with BZ, ABP and (+)-BP-7,8-diol were performed in triplicate with oCOX-1 and -2 utilized as positive controls; additional substrates were tested in duplicate with only oCOX-1 utilized as positive control.

Protein binding of BZ was examined in a similar manner, with the exception that reactions were stopped by the addition of 20 µl concentrated HCl. The tubes were then centrifuged at low speed (1000 g) to obtain the precipitated protein. This procedure minimized contamination with DNA (which will sediment with the precipitated protein at higher speeds). The supernatant was removed, and the protein was resuspended in 50 mM potassium phosphate buffer (pH 7.4) and extracted five times with an equal volume of ethyl acetate. These extracts were analyzed by liquid scintillation counting. Protein was again precipitated by addition of HCl and centrifuged. Precipitated protein was solubilized in 0.1 ml water for liquid scintillation counting.

Statistics

DNA binding values obtained were analyzed by Student’s t-test using Microsoft Excel.

Results

Activation of 4-aminobiphenyl, (+)-BP-7,8-diol and benzidine

All four cyclooxygenases (oCOX-1, oCOX-2, hCOX-1 and hCOX-2) activated ABP, (+)-BP-7,8-diol and BZ to DNA binding species (Figure 2). The hCOX enzymes displayed equal or greater activation of ABP and (+)-BP-7,8-diol as
Carcinogen substrate specificity of hCOX-1 and -2

Experiments were undertaken to determine whether protein binding was the cause of the reduced DNA binding for hCOX isozymes. As shown in Figure 3, a dose-dependent decrease was seen when 0.2 and 0.4 mg of hCOX-1 microsomes were added to 750 ng of oCOX-1. This blocking of DNA binding was mimicked by the addition of 0.2 and 0.4 mg of bovine serum albumin (BSA). As a control, MOCA was also investigated as a substrate in the BSA experiments. It was determined that the addition of BSA had no significant effects on COX-catalyzed MOCA–DNA binding (results not shown).

Next, as indicated in Materials and methods, reactions containing either 750 ng of oCOX-1 and 0.4 mg BSA, or 0.4 mg hCOX only, were performed (results not shown). These experiments demonstrated a significant amount of BZ binding to both the COX microsomal proteins and the BSA (2.2 nmol and 570 pmol, respectively). Even if residual, adducted DNAoCOX-1, oCOX-2, hCOX-1 and hCOX-2. Substrates are shown in: (A) ABP, (B) (+)BP-7,8-diol and (C) BZ. Note the logarithmic scale for BZ. Values shown are averages (n = 3) with standard deviation.

Comparison of hCOX-1 and -2 with oCOX-1 and -2

Activation of IQ, MeIQx, PhIP, MOCA and (+)-BP-7,8-diol

Ovine COX-1 and the hCOX isozymes demonstrated the ability to activate IQ, MeIQx, PhIP, MOCA and (+)-BP-7,8-diol. For the hCOX-catalyzed reactions, those containing MOCA led to the highest levels of DNA binding observed (Figure 4). There was also a difference in the binding levels catalyzed by hCOX-1 as compared with hCOX-2, with hCOX-2 utilizing MOCA as a reducing substrate and activating it to an electrophile at nearly twice the level of hCOX-1 (P = 0.007). MeIQx was effectively utilized by hCOX as a reducing substrate as demonstrated by levels of DNA binding nearly equivalent to those of ABP. (+)-BP-7,8-diol was bioactivated by hCOX-2 at the same level as the purified (+)stereoisomer alone, but at a slightly higher average binding level (23% greater; P = 0.03) for the (+)-stereoisomer as compared with the (+)-racemic mixture by hCOX-1. From

Fig. 3. Effect of protein on BZ–DNA binding. 750 ng of oCOX-1 was used to activate BZ. This binding was quenched by the addition of 200 then 400 µg of hCOX-1. hCOX-1 (400 µg) catalyzed BZ activation is also shown. Additionally, as discussed in Materials and methods, 200 and 400 µg of BSA were utilized to replicate the effect of hCOX-1 microsomal protein addition to 750 ng of oCOX-1.

Fig. 2. COX-catalyzed arachidonic acid-dependent substrate activation by oCOX-1, oCOX-2, hCOX-1 and hCOX-2. Substrates are shown in: (A) ABP, (B) (+)-BP-7,8-diol and (C) BZ. Note the logarithmic scale for BZ. Values shown are averages (n = 3) with standard deviation.

*P = 0.006, #P < 0.05 for comparisons between hCOX-1 and -2 and between oCOX-1 and -2, respectively. In untransfected cell enzyme preparations, arachidonic acid dependent binding was <0.1 pmol/mg DNA.
these results, it is apparent that hCOX-2 activates (+) and
(−)BP-7,8-diol to the same degree, while hCOX-1 apparently
displays a greater activation of the (+)-stereoisomer.

Additionally, to insure that lipoxygenases were not
contributing to the activation of the BP diols, control reactions
were carried out containing 30 μM nordihydroguaiaretic acid,
a selective lipoxygenase inhibitor, with no apparent effect on
substrate binding values (data not shown).

MeIQx appeared to be readily bioactivated by oCOX-1, hCOX-1 and hCOX-2. In contrast, the activation of PhIP and
IQ in this test system by the COX enzymes were at levels just
above the limits of detection (Figure 4). An exception was
oCOX-1, which showed appreciable activation of IQ.

Discussion

Human microsomal PGHS, which may have contained either
COX-1 or -2, has been shown to be capable of activating
a variety of substrates (23,25,26). Thus, the activation of
dietary and environmental carcinogens by these extra-hepatic
enzymes may play a significant role in normal tissue, or in
tumors such as colon, lung, breast and skin, all of which

Fig. 4. COX-catalyzed arachidonic acid-dependent DNA binding. (A) Heterocyclic amine
substrates are compared with (±)-BP-7,8-diol and MOCA. Values obtained for the activation of
PhIP, (±)-BP-7,8-diol, IQ, MeIQx and MOCA by hCOX-1 and -2 are shown with corresponding
oCOX-1 positive controls. Note, the left y-axis is for all substrates except MOCA and the right
y-axis is for MOCA. Values shown are averages (n = 2) of duplicate experiments where values
were within 5–10% of each other. (B) Values for hCOX-1 and -2 compared for all substrates
tested (left y-axis for all substrates except MOCA, right y-axis for MOCA).
have been shown to express either COX-1 or -2, or both (11–15). Our study has now shown that both hCOX isoforms are capable of activating several known, or suspected human carcinogens.

The oCOX enzymes, utilized primarily as positive control standards, exhibited significant differences in activation for BZ, ABP and (+)BP-7,8-diol in comparison with hCOX. For each substrate, this difference in activation was similar. The fact that little difference in activation between the hCOX enzymes was observed for most substrates indicates that the large differences seen between oCOX-1 and -2 may be due to factors other than enzyme–substrate affinity, such as differences in stability between isoforms. Regardless, the values obtained are useful for comparison with the hCOX activation levels.

The level of DNA binding for MOCA was greater by far than for any substrate tested and a sizeable body of evidence exists implicating this compound in human and animal carcinogenesis (33). MOCA is a widely used industrial chemical that is not found in nature and it has been estimated that since 35 000 workers are potentially exposed to this substance (34). It is used as a curing agent for isocyanate-containing polymers (polyurethane) and also as a curing agent in epoxy and epoxy-urethane resin blends. Although epidemiological evidence of MOCA contributing to human cancers is limited, there is an abundance of animal data documenting hepatic and lung cancers in treated rats and mice (33) and urinary bladder tumors in dogs (35,36). Additionally, it has been shown that MOCA is present in the urine of workers exposed to this compound (34), and that acute exposure to MOCA results in the formation of DNA adducts in exfoliated human urothelial cells (37). COX enzymes are known to be present in the urinary bladder (20,38). As shown in Figure 4, the hCOX isoforms activate MOCA at a level appreciably greater than oCOX-1; therefore, previous work utilizing oCOX would not have predicted, as has been demonstrated here, that MOCA is highly activated by hCOX isoforms, resulting in DNA covalent binding.

ABP is also a readily utilized reducing substrate for both COX isoforms; this near ubiquitous carcinogen is found in a variety of sources, most notably cigarette smoke. This finding and the activation of the food-borne heterocyclic amine MeIQx, indicate a mechanism whereby activation of these compounds may occur in normal (COX-1) as well as in neoplastic (COX-1 and -2) colonic mucosa. Previous work (25) has demonstrated significant activation of IQ in hCOX assays and little or no PhIP activation. The difference between the findings of London et al. (25) and those recorded here pertaining to IQ activation may be partially due to the fact that a 5-fold greater substrate concentration (20 versus 100 gM) was utilized in their experiments. Additionally, PhIP did not appear to function as a reducing substrate in this previous work, whereas it did in the system described here (though at a very low level). This discrepancy may be due to enzyme preparation. In the previous experiments, the enzyme was prepared by the sonication of transfected COS-1 cells, and sonication can introduce oxygen radicals and thermally denature proteins, possibly disrupting the very low level of PhIP activation.

The protein binding of BZ observed here is consistent with the activated species of BZ. The two-electron oxidation product of BZ, produced by the COX enzymes, is BZ diimine, which is a relatively soft electrophile (39). The ample supply of thiol and amine targets in the recombinant human microsomal preparations may divert activated species from DNA adduct formation. These results are in agreement with work done by Eling et al. that demonstrated the need for acetylation of BZ for mutagenesis (40,41). In the BSA-containing reactions, which were used as a model of the hCOX reactions, the same level of inhibition and binding was not seen (although appreciable levels of inhibition are present). This is attributable to the fact that BSA contains only one free thiol per molecule, which is presumably the residue where most of the BZ diimine is binding, while the recombinant microsomes may contain proteins with several free thiols (or other possible sites, e.g. lysine) giving the microsomes a greater quenching capacity than that of BSA.

The activation of the BP metabolite, BP-7,8-diol, by the COX isoforms may be especially relevant to skin, lung and colon carcinogenesis, as these sites receive direct exposure to polycyclic aromatic hydrocarbons (PAHs; occupational, environmental, smoking, diet). Moreover, these tissues contain both cytochromes P450 1A1 and P450 1B1 (reviewed in ref. 42), which readily convert BP to its 7,8-diol; and have each been shown to express COX enzymes that can further activate this metabolite to form DNA adducts.

The work done here establishes carcinogen activation by hCOX enzymes to covalently bind to DNA in vitro. Several studies have been conducted in vivo in animal models that support a role for COX in chemically-induced carcinogenesis. The co-administration of docosahexaenoic acid (DHA, C22:6, ω-3), which is thought to inhibit COX and lipoxygenase metabolism in vivo, has been shown to block chemically-induced carcinogenesis in the colon (43–45). Specifically, work done by Takahashi et al. (46) utilizing a rat model administering DHA and PhIP has demonstrated this inhibition. After 4 weeks of treatment, the rats receiving DHA in addition to PhIP had 53% fewer aberrant crypt foci (putative preneoplastic lesions) than the rats administered PhIP only. In a murine model of familial adenomatous polyposis (APC5710), constitutive high-level expression of COX-2 is observed (47). In this murine model, heterocyclic amines present in high-temperature cooked meats were shown to stimulate polypl growth (48); while DHA significantly reduced the number of polyps (49).

The activation of several substrates, demonstrated here utilizing human enzymes, along with the abundant animal data indicate a role for hCOX in human chemically-induced carcinogenesis. As has been shown previously, COX-1 and -2 are present in several extra-hepatic tissues, most notably the colon where cytochrome P450 levels are low and COX is known to play a role in carcinogenesis; the presence of active enzyme and the near certainty of exposure to many of these tested compounds would seem to ensure a role for COX in chemically-induced human carcinogenesis.

References


