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ACTIVATION OF 2-AMINOFLUORENE BY PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE-2[1,2]

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Prostaglandin endoperoxide H synthase is the key enzyme in the conversion of arachidonic acid to tissue prostanoids. Two isoforms of prostaglandin endoperoxide H synthase have been identified: PHS-1 is constitutively expressed in most tissues under normal physiological conditions and PHS-2 is expressed in response to inflammatory agents, tumor promotors, and other agents related to mitogenesis. Previous work demonstrated that PHS-1 can activate arylamine carcinogens. We report here that PHS-2 can also activate an arylamine carcinogen to form DNA adducts. This is shown by: (1) use of purified ovine PHS-2 to form DNA adducts; (2) increased DNA adduct formation, PHS-2 mRNA, and PHS-2 protein after treatment of HUVEC cells with the PHS-2 inducer PMA; and (3) transient expression of PHS-2 cDNA in COS-1 cells gave rise to both elevations of PHS-2 enzyme protein and DNA adduct formation. Finally, two PHS inhibitors, aspirin and indomethacin, showed significant inhibition of PHS-2-mediated DNA adduct formation.

Most carcinogenic chemicals encountered in our environment require metabolic activation to form DNA-binding ultimate carcinogens which cause DNA damage and initiate carcinogenesis. The cytochrome P450 system of monooxygenases is the metabolic pathway most commonly involved in carcinogen activation, especially in the liver. However, oxidative reactions catalyzed by peroxidases and other enzymes occur in many extrahepatic tissues. Since the discovery of prostaglandin endoperoxidase H synthase in the extrahepatic tissues, there has been great interest

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Abbreviations:

AA: arachidonic acid; AF: 2-aminofluorene; DMEM: Dubelco's modified Eagle medium; D-PBS: Dubelco's phosphate-buffered saline; FBS: fetal bovine serum; G3PDH: glyceraldehyde 3-phosphate dehydrogenase; HUVEC: human umbilical vein endothelial cells; PHS-1: prostaglandin endoperoxide H synthase 1 = COX-1: PHS-2: prostaglandin endoperoxide H synthase 2 = COX-2: PMA: phorbol 12-myristate 13-acetate, SDS: sodium dodecyl sulfate; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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in evaluating carcinogen activation by this system [1-4]. PHS contains both cyclooxygenase and peroxidase activities which catalyze the two steps in the conversion of arachidonic acid to prostaglandin H_2. Other enzymes then convert prostaglandin H_2 to tissue prostaglandins, thromboxanes, and prostacyclins. During the oxidation of arachidonate to prostaglandins, free radicals which can activate xenobiotics to biologically reactive intermediates are produced. The activated xenobiotics, in turn, can bind covalently to macromolecules such as DNA. Investigations of activation of carcinogenic arylamine compounds have indicated the involvement of PHS-mediated activation [2,5-7]. DNA adducts consistent with PHS-mediated activation of arylamines such as AF have been linked to susceptibility to bladder cancer [5,7].

Recently, it has been discovered that there are two isozymes of PHS: PHS-1 and PHS-2 [4]. Both isoforms have been purified, characterized, cloned, and functionally expressed. Although the two isozymes are similar in their protein structures as well as the general reactions they carry out, the regulation of expression is quite different between them. While PHS-1 is constantly expressed within a somewhat variable range, PHS-2 can be greatly induced by a wide variety of physiological or pharmacological agents such as inflammatory mediators (*e.g.* cytokines), growth factors, and tumor promotors (*e.g.* phorbol esters). Without induction, PHS-2 is barely detectable in most tissues. The earlier work on PHS-mediated carcinogen activation has been done with enzyme from sheep or bull seminal vesicles which is now known to be PHS-1 [2,5,7]. Therefore, information on the role of PHS in the bioactivation of carcinogens should be reevaluated in light of the new knowledge about PHS-2.

In this paper, we have tried to evaluate the *in vivo* and *in vitro* AF-DNA adduct formation by PHS-2 as well as PHS-1 from different sources of the enzymes: from purified ovine seminal vesicle PHS-1 and placental PHS-2; from human PHS-1 and PHS-2 cDNA-directed expression in COS-1 cells; and from induced PHS-2 in HUVEC. Finally, we have observed the inhibitory effect of NSAIDs (non-steroid anti-inflammatory drugs) on AF-DNA adduct formation mediated by PHS-2.

Materials and Methods

Materials: HUVEC and EGM medium were supplied by Clonetics (San Diego,CA), COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD), human cDNAs of COX-1 and COX-2 [8] were kindly provided by Dr. Hla, (Holland Laboratory, American Red Cross, Rockville, MD) in the eukaryotic expression vector, pcDNAI/Neo. Another human cDNA of COX-2 [9] was given generously by Dr. Prescott (Department of Internal Medicine and Biochemistry, University of Utah, Salt Lake City, UT) in plasmid bluescript (PBS plasmid). PMA was from Sigma (St. Louis, MO). [^{3}H]AF, specific activity 247 mCi/mmol and purity >93%, was supplied by Chemsyn Science Laboratory (Lenexa, KS). G3PDH cDNA probe was from Clontech (Palo Alto, CA). Zeta-probe GT membrane for northern blotting and nitrocellulose membrane for western blotting were purchased from BioRad (Hercules, CA), western blotting ECL kit and [^{32}P]dATP from Amersham (Arlington Heights, IL). Rabbit anti-ovine COX-1 and anti-human COX-2 antibodies as well as arachidonic acid (AA) were from Caymen (Ann Arbor, MI).

Subclonings of human COX-1 and COX-2 and the cDNA probe preparation: The human COX-1 and COX-2 in pcDNA I/Neo vector from Dr. Hla were first subcloned separately into Hind III/XhoI sites of pcDNA 3.The final construction of COX-1 was named pcDNA

3.COX-1. The subcloned COX-2 in pcDNA3 was used further to construct the native sequence. To repair the mismatch between the construct of this human COX-2 sequence and the native sequence in COX-2 cDNA [9,10], a 1.75 kb EcoRI/EcoRV fragment of the 5'-region subcloned in pcDNA 3 of this cDNA was further replaced with the corresponding EcoRI/EcoRV fragment of human COX-2 from Dr.Prescott. Therefore, the insert of pcDNA 3.COX-2, the final construction of human COX-2 in the pcDNA 3 vector, consisted of 1812 bp in the coding region, and 87 bp and 20 bp in the 5'- and 3'-noncoding regions, respectively, matching perfectly the DNA sequences [9] as confirmed by DNA double strand sequencing in both directions. The whole cDNAs of human COX-1 and COX-2 with the lengths of about 1.8 and 1.85 kb, respectively, were used as the cDNA probes for northern blotting after digestion with Hind III and XhoI.

Human COX-1 and COX-2 cDNA expressions in COS-1 cells: The transfection of plasmid DNAs, pcDNA.COX-1 and pcDNA.COX-2, were done by the DEAE-Dextran method [11] followed by chloroquine treatment [12]. Briefly, semiconfluent cultures of COS-1 cells were transfected with the recombinant plasmids (15 μg/10 cm dish) in DEAE-dextran (500 μg/ml DMEM) for 2 hours. As control, cells transfected with non-recombinant pcDNA 3 were included in every transfection. After treatment with chloroquine (52 μg/ml) in DMEM for 3 hours the cells were incubated for 48 hours in DMEM-10 % FBS. Two alternatives procedures with the cells were followed. 1. For the *in vivo* AF-DNA adduct formation assay, [^{3}H]AF and AA were added to the medium of DMEM-10 % FBS to final concentrations of 5 μM and 25 μg/ml, respectively. After a further incubation of 12-17 hours, the cells were harvested by scraping with a disposable cell scraper and resuspended in 1 x D-PBS buffer at 4^o. After centrifugation at 1500 x g for 5 minutes at 4^o, the cell pellet was washed two times to remove media. 2. For the *in vitro* AF-DNA adduct formation assay, cells without AF or AA treatment were harvested as above.

Genomic DNA extraction and determination of radioactive labelling: The procedure for genomic DNA extraction was followed as described [13]. Briefly, the cells was lysed in a lysis buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5 % SDS and 0.1 mg/ml proteinase K) at 50^o for 15-18 hours. The sample was then extracted with an equal volume of phenol/chloroform. After centrifugation, the aqueous phase was transfered to a new tube and 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol was added. The DNA was pelleted by a centrifugation at 1,700 x g for 2 minutes. RNA was removed from the DNA sample by an incubation in 0.1% SDS containing 1 μg/ml RNase (DNase free), followed by another organic extraction and ethanol precipitation. The DNA pellet was finally dissolved in TE buffer (pH 8.0). Radioactivity of [^{3}H]AF bound to the genomic DNA was quantitated by scintillation counting as described [5].

***In vitro* activation of AF by purified ovine PHS-1 and PHS-2:** The procedure was similar to that reported previously [5,14] except additional reactions using PHS-2 were carried out. Each reaction contained 12.5 μCi (final concentration 50 μM) [^{3}H]AF, 1 ml calf thymus DNA (2 mg/ml in K-PO_4 pH 7.4), 1 μM hematin, and 0, 100, or 300 units of ovine seminal vesicle PHS-1 or ovine placental PHS-2. Reactions were started with arachidonic acid (final concentration 110 μM) and after 5 min at 37^o were stopped and DNA precipitated with 5 ml ethanol and 50 μl phenol. After 30 min at -20^o the tubes were centrifuged, the DNA pellet was recovered and washed with 70% ethanol and then redissolved in 1 ml of 50 mM K-PO_4 (pH 7.4). Aliquots were taken for determination of radioactivity by scintillation spectrometry and of DNA by uv absorbance. Repeated precipitation and redissolution were carried out until specific radioactivity of the DNA remained constant (4 or 5 cycles).

***In vitro* activation of AF by human PHS-1 and PHS-2 from transfected COS-1 cells:** COS-1 cells were transfected with pcDNA.COX-1, pcDNA.COX-2, or pcDNA3 as described. The harvested cells were washed and suspended in 50 mM K-PO_4 (pH7.4) and sonicated. From 100 to 300 μl of the cell sonicate were used as enzyme source in reactions similar to those described for purified ovine PHS. In addition, incubation with sonicates of pcDNA.COX-2 transfected cells were also carried out with 50 μM aspirin or 100 μM indomethacin (final concentrations) added 3 min before starting the reactions with arachidonic acid. AF-DNA adduct formation was determined as for the ovine PHS experiments.

Total RNA preparation and northern blotting: Total RNA was prepared from the cultured cells by following a single step method [15]. For northern blotting, the protocol for Zeta-probe GT membrane from the manufacturer was followed. Total RNA (10-15 μg) was separated in a 2.2 M formaldehyde agarose gel. The RNA was then transferred onto a Zeta-probe GT membrane. Prehybridation and hybridization were conducted in 0.25 M Na_2HPO_4 (pH 7.2)/7 % SDS. Random labeling with [^{32}P]dATP was used to radiolabel the cDNA probes. Typically, 0.5-3 x 10^6 cpm per ml hybridization buffer were applied for the hybridization with the three human probes, G3PDH, COX-1 and COX-2. The radioautography was done using Kodak XAR-5 film at -70°.

Western blotting: SDS-PAGE was done as described by Laemmli [16] with 10-12% acrylamide in the separating gel. Typically, 30-100 μg sample of cell homogenate was loaded in each lane. The proteins were transferred onto Trans-blot nitrocellulose membrane (0.45 μm) with 100 mAmp for 12-18 hours in the transfer buffer (39 mM glycine, 48 mM tris-HCl, 0.0375% SDS) [17]. The western blot was done with enhanced chemiluminescence detection using an Amersham ECL kit. Briefly, the membrane was blocked with 5% bovine serum albumin and incubated at 4° overnight with 2,000 x diluted anti-COX-2 antibody or anti-COX-1 antibody. The secondary antibody was peroxidase-labeled donkey anti-rabbit IgG and was incubated at room temperature at a dilution of 2,000. The sheet was further incubated with a fresh mixture of the detection agents 1 and 2 included in the kit. The filters were immediately blot-dried and exposed to XAR-5 Kodak films.

Results

***In vitro* bioactivation of AF by purified ovine COX-1 and COX-2:** The values for the activities of ovine seminal vesicle COX-1 and ovine placental COX-2 were supplied by Caymen. The bioactivation of AF is shown in Figure 1. At the two levels of enzyme tested (100 units and 300 units), both COX-1 and COX-2 activated AF to a DNA-binding form. The amount of adduct

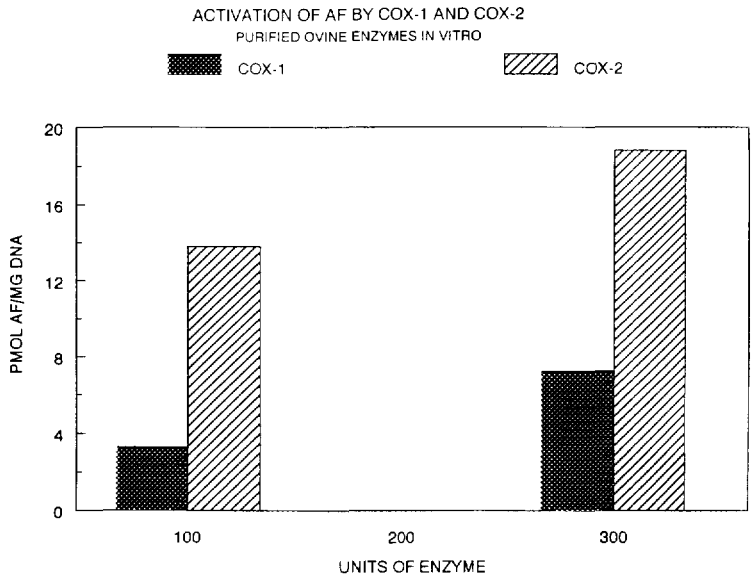

Figure 1. *In vitro* activation of AF by purified ovine PHS-1 and PHS-2. Two different amounts of each enzyme were used as described in the Materials and Methods.

formed was dependent on the amount of enzyme added. PHS-2 gave 2-4 fold more AF-DNA adduct than PHS-1 per unit of cyclooxygenase activity.

***In vivo* DNA adduct formation by PHS-2 induced with PMA in HUVEC:** PMA induced PHS-2 but not PHS-1 [reviewed in 4]. This was confirmed experimentally as shown in Figure 2A and 2B. In the sample treated with PMA, an increased transcription of PHS-2 mRNA and, at the same time, an elevated level of PHS-2 protein were observed compared to the control samples: no bands were detected in the untreated control sample. The induced protein gave three bands (74, 72, and 70 kD), consistent with a previous report [18]. At the same time, there was no change for PHS-1 in either mRNA or protein levels (Figures 2A and 2B). Accordingly the nearly three-fold increase in covalently bound radioactivity in figure 2C, the DNA sample from HUVEC

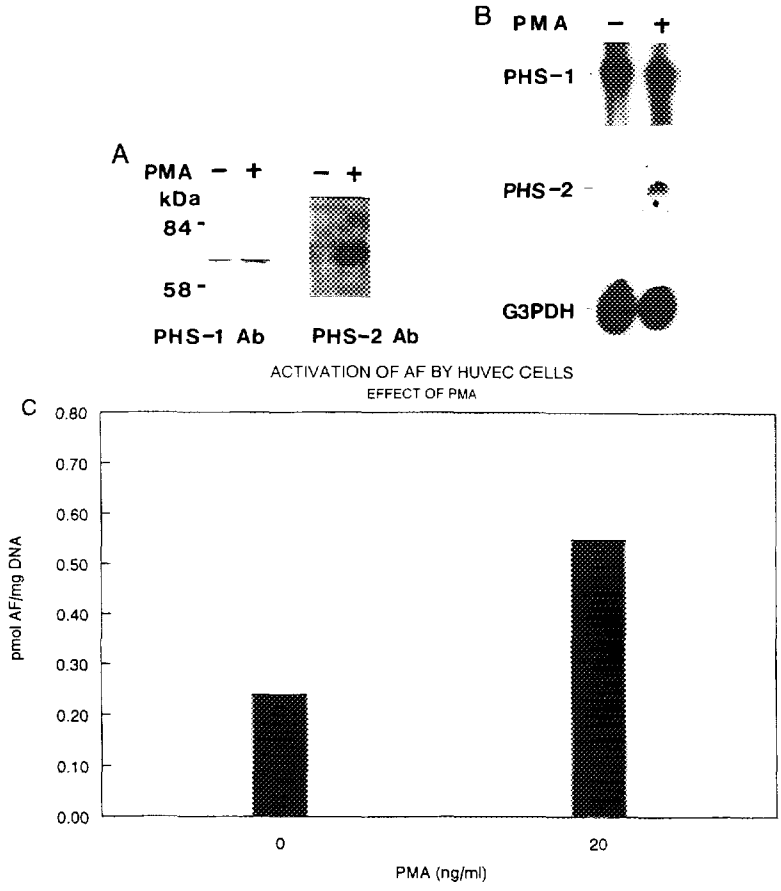

Figure 2. Effect of treatment with PMA (20 ng/ml) on PHS-1 and PHS-2 in HUVEC. **A**: Detection of immunoreactive PHS-1 and PHS-2 by western blots. 50 μg of cell homogenate was loaded in each lane. PMA treatment did not alter the appearance of PHS-1 immunoreactive protein, but did lead to the appearance of 3 immunoreactive bands corresponding to PHS-2. **B**: mRNA levels of PHS-1 and PHS-2 determined by Northern blot. A probe for G3PDH was used as a control. 20 μg total RNA was loaded in each lane. mRNA for PHS-2 was seen only after PMA treatment. **C**: Formation of AF-DNA adducts after induction with PMA in HUVEC cells.

cells induced by using PMA, is attributed to the induction of PHS-2 by PMA. This experiment was repeated once and comparable data were obtained.

***In vitro* activation of AF by PHS-1 or PHS-2 cDNA-directed expression in COS-1 cells:** To further evaluate the role of human COX-2 as well as COX-1 in the bioactivation of AF, the cDNAs encoding human COX-1 and COX-2 in pcDNA 3 vector were transfected into COS-1 cells. The expression of the enzymes was confirmed by western blots (Figure 3A): wild-type plasmid sample gave no band, but samples transfected with human COX-1 and COX-2 cDNAs showed 70 kD and 74 kD bands, respectively. The *in vitro* DNA adduct assay using AF as the co-substrate and cell sonicate as enzyme source, showed that cells transfected with human COX-2 as well as with COX-1 were able to activate AF to form DNA adducts (Figure 3B).

Inhibition of PHS-2-mediated activation of AF by aspirin and indomethacin: In this experiment, we examined the ability of aspirin and indomethacin to inhibit PHS-2 activation of AF. The cell sonicate from COS-1 cells transfected with COX-2 cDNA was used as the enzyme

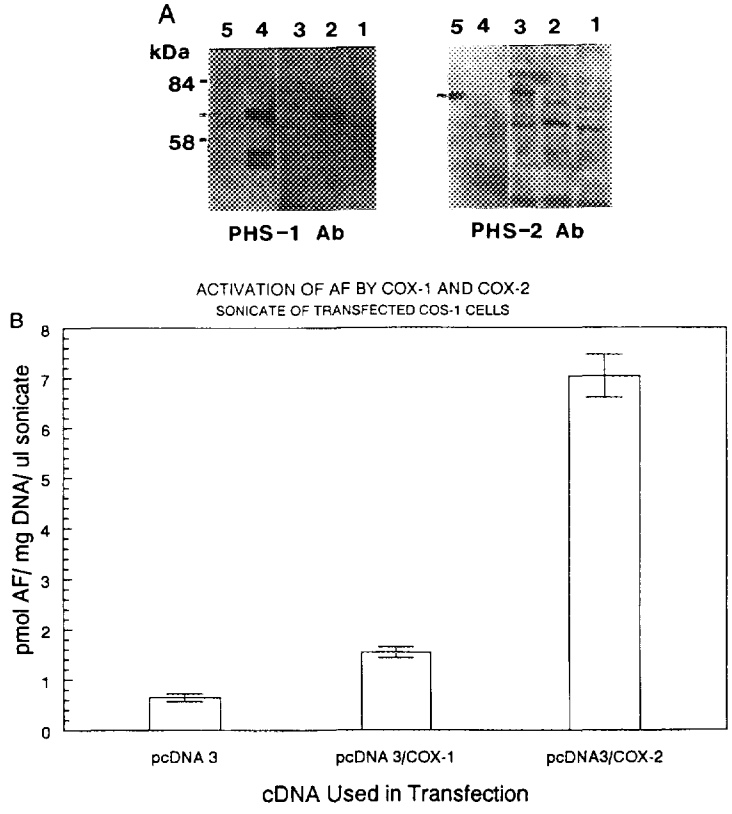

Figure 3. **A**: Western blots of COS-1 cell samples transfected with three plasmid constructs. 500 ng of purified ovine PHS-1 (lane 4) or PHS-2 (lane 5) were included as references. 30 μg of cell homogenate from each sample was loaded per lane (1-3). Cells transfected with PHS-1 gave a single band of protein at 70 kD (lane 2) while a 74 kD band was observed with PHS-2 (lane 1) and no immunoreactive bands were seen with pcDNA 3 (vector control transfected cells, lane 1). **B**: AF-DNA adduct formation catalyzed by sonicates of the transfected cells.

source. Treatment with the two NSAIDs inhibited AF-DNA adduct formation by approximately 60% for aspirin and 70% for indomethacin (Figure 4).

Discussion

Many of the previous studies of carcinogen activation by PHS were carried out before the discovery of PHS-2, the inducible isozyme of PHS. The majority of experiments used enzyme at various degrees of purification from seminal vesicles (an excellent source of PHS-1) or with microsomes from tissues at unknown levels of PHS-2 induction [5,14]. We now know that many different classes of inducing agents can very significantly affect the level and activity of PHS-2 [4]. Since PHS-2 was not considered in earlier studies, the role of this enzyme in the activation of carcinogens could not be determined.

In this paper we have shown for the first time, that PHS-2 is capable of activating the carcinogenic arylamine AF to a DNA-binding form. We have shown this by *in vitro* incubation of purified ovine placental PHS-2 with exogenous DNA, by *in vitro* incubation of sonicate from COS-1 cells transfected with human PHS-2 cDNA, and by inducing PHS-2 in cultured HUVEC and observing AF-DNA adduct formation in the DNA of the HUVEC. At the same time we have confirmed the induction of PHS-2 mRNA and PHS-2 immunoreactive protein in HUVEC as well as the PHS-2 cDNA directed expression of PHS-2 immunoreactive protein in transfected COS-1 cells.

Numerous studies have shown that drugs which inhibit PHS activity decrease the incidence of gastrointestinal cancers in animals [19] and colon and rectal cancers, in particular, in humans [20,21]. The PHS-inhibiting drugs, mainly NSAIDs, have been shown to act on both

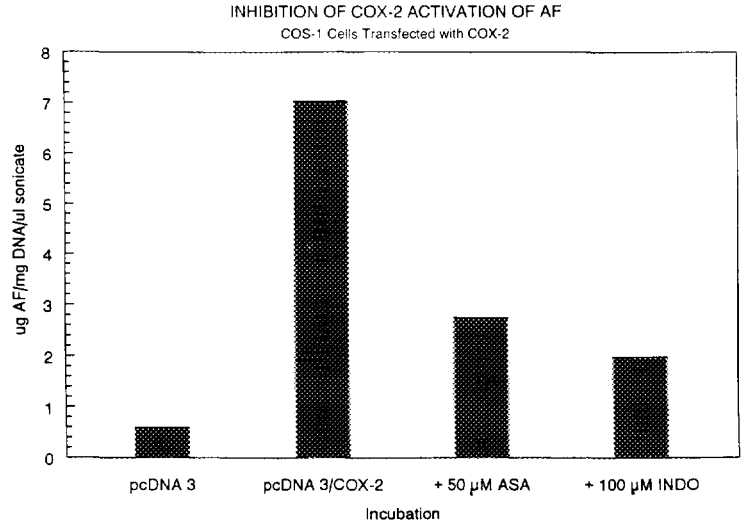

Figure 4. Inhibition by aspirin (ASA) and indomethacin (INDO) of PHS-2 activation of AF in the sonicates of COS-1 cells transfected with COX-2 cDNA. The sample, indicated as pcDNA 3 is the sample of cells transfected with the wild-type plasmid (control) as described in Materials and Methods.

PHS-1 and PHS-2 with some differences in their relative effectiveness [10,22,23]. Thus, until recently, the role of PHS-2 in carcinogenesis could not be evaluated directly. Two recent studies have indicated the importance of PHS-2 in carcinogenesis. Eberhart *et al.* [24] found that while PHS-1 mRNA did not differ between normal and cancerous human colonic mucosa, PHS-2 mRNA was increased as much as 50-fold in colorectal adenocarcinomas. Another study of PHS-1 and PHS-2 protein expression in human colon cancer showed increased expression of PHS-2 protein in cancerous tissue and a decrease in PHS-1 protein compared to normal tissue from the same individual [25].

We have now shown that PHS-2 can activate an arylamine carcinogen to form DNA adducts and that the adduct formation can be inhibited by aspirin and indomethacin. Our results, taken together with the epidemiological studies [20,21] and the studies of Eberhart and Kargman [24,25], suggest that the beneficial effects of NSAIDs in preventing colon and other cancers may be due to inhibition of PHS-2.

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