Short Communication

ROLE OF HUMAN CYCLOOXYGENASE-2 IN THE BIOACTIVATION OF DAPSONE AND SULFAMETHOXAZOLE

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ABSTRACT:

Sulfamethoxazole (SMX) and dapsone (4,4′-diaminodiphenylsulfone, DDS) are believed to mediate their adverse effects subsequent to bioactivation to their respective arylhydroxylamine and arynitroso metabolites, resulting in covalent adduct formation with intracellular proteins. Various bioactivating enzymes, such as cytochromes P450 and myeloperoxidase, have been shown to be capable of catalyzing the N-oxidation of these compounds. We assessed the role of human cyclooxygenase-2 (COX-2) in the metabolism and subsequent adduct formation of DDS and SMX using recombinant human COX-2. Using an adduct-specific enzyme-linked immunosorbent assay, we found that the complete enzyme system gave rise to covalent adducts. However, the nonspecific COX inhibitor indomethacin did not reduce the amount of covalent adduct formed. Formation of the arylhydroxylamine metabolites was demonstrated via high performance liquid chromatography coupled with UV absorption. Metabolite formation was found to be secondary to the H₂O₂ in the incubation mixture and was not enzyme-mediated. Hence, COX-2 does not play a direct role in the bioactivation of these parent drugs to their arylhydroxylamine metabolites.

Administration of sulfonamide antimicrobials such as sulfamethoxazole (SMX) and the sulfone dapsone (4,4′-diaminodiphenylsulfone, DDS) has been associated in humans with hypersensitivity reactions that include fever, skin eruptions, hepatotoxicity, and blood dyscrasias (Dujoine et al., 1967; Rieder et al., 1989). The mechanism of sulfonamide hypersensitivity is not well understood, but has been hypothesized to be secondary to the generation of the reactive oxidative metabolites such as SMX-hydroxylamine (SMX-NOH) and DDS-hydroxylamine (DDS-NOH) and their respective nitroso derivatives (Svensson, 2003). The arylhydroxylamine metabolites of DDS and SMX, unlike the parent sulfonamides, are cytotoxic to a variety of cells in vitro and have been shown to generate reactive oxygen species (Rieder et al., 1995; Reilly et al., 2000; Vyas et al., 2005). The parent drugs and their arylhydroxylamine metabolites have been demonstrated to haptenize cellular proteins, which may lead to immune-mediated cutaneous reactions (Manchanda et al., 2002; Naisbitt et al., 2002; Roychowdhury et al., 2005). Thus, the bioactivation of these arylamine xenobiotics to their respective arylhydroxylamine metabolites may be the first and most important step in the initiation of these reactions.

Previous studies have demonstrated the ability of various oxidizing enzymes, including CYP2C9, CYP2E1, and CYP3A4, as well as myeloperoxidase to bioactivate arylamines in vitro (Cribb et al., 1990, 1995; Mitra et al., 1995; Cashman et al., 1999; Winter et al., 2000). Cyclooxygenases (COXs), or prostaglandin H synthase, have also been shown to bioactivate heterocyclic amines to their hydroxylamine metabolites (Liu and Levy, 1998). Procainamide, an arylamine anti-arrhythmic agent, has also been found to be oxidized to its arylhydroxylamine and arynitroso metabolites by COX-2 (Goebel et al., 1999). The N-oxidation of 4-chloroaniline has also been reported to be mediated by COXs (Golly and Hlavica, 1985). Based upon these observations, we tested the hypothesis that COX-2 may bioactivate SMX and DDS, resulting in protein haptenation.

Materials and Methods

Materials. Unless specified otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA). DDS and SMX hydroxylamine metabolites were synthesized as described previously (Vyas et al., 2005). Human recombinant COX-2 was obtained from Cayman Chemical (Ann Arbor, MI). Rabbit antiserum was raised against SMX- and DDS-keyhole limpet hemocyanin conjugates, and specificity was assessed as described previously (Reilly et al., 2000). Goat anti-rabbit antibody conjugated with alkaline phosphatase was purchased from Invitrogen (Carlsbad, CA). Microtiter ELISA plates (96-well) were obtained from Rainin Instruments (Woburn, MA).

Adduct Formation of DDS by Human Recombinant COX-2. An incubation mixture containing COX-2 (100 units), arachidonic acid (1 mM), hematin (1 μM), EDTA (5 mM), and H₂O₂ (1 mM) in Tris-HCl buffer (50 mM, pH 8.00), with and without INDO or DDS (100 μM each), was incubated for 1 h at 37°C. A control incubation containing only buffer was also included to account for nonspecific binding to the microtiter plate. After 1 h of incubation, the reaction mixture was left overnight at 4°C for complete adhesion of protein to the microtiter plate. In addition, a DDS-bovine serum albumin adduct was added to a set of wells at this time to serve as a positive control for adduct detection. After 24 h, covalent adducts were determined by an adduct-specific ELISA as described previously (Reilly et al., 2000).

Determination of COX-2-mediated Arylhydroxylamine Formation of DDS and SMX via High Performance Liquid Chromatography (HPLC). DDS or SMX (800 μM) was added to the incubation mixture described above, with and without COX-2 (100 units), for 1 h at 37°C. Ascorbic acid (1 mM) was included in all incubations to stabilize the arylhydroxylamine formed.

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ABBREVIATIONS: SMX, sulfamethoxazole; DDS, 4,4′-diaminodiphenylsulfone, dapsone; DDS-NOH, dapsone hydroxylamine; INDO, indomethacin; SMX-NOH, sulfamethoxazole hydroxylamine; COX, cyclooxygenase; ELISA, enzyme-linked immunosorben assay; HPLC, high performance liquid chromatography.
After 1 h, the reaction was terminated by addition of 3 ml of ethyl acetate and the arylhydroxylamine metabolites determined as described previously (Reilly et al., 2000). As a positive control to assure the catalytic activity of COX-2 under these incubation conditions, reactive oxygen species generation was determined via the oxidation of the fluorescent dye 2',7’-dichlorodihydrofluorescein (5 μM), as we have previously described (Vyas et al., 2005).

**Determination of H$_2$O$_2$-Mediated Arylhydroxylamine Formation of SMX or DDS via HPLC.** SMX or DDS (800 μM) was incubated in Tris-HCl buffer (50 mM, pH 8.00) and ascorbic acid (1 mM) with increasing concentrations of H$_2$O$_2$, ranging from 0.01 μM to 10 mM. The incubation mixtures were kept for 1 h at 37°C. After 1 h, the metabolites formed were extracted and quantified via HPLC.

**Statistical Analysis.** Data are presented as mean (S.D.). Statistical comparisons between two groups were made using either Student’s $t$ test (parametric method) for normalized data or Friedman’s rank sum test (nonparametric method) for the data that did not pass the normality test. For the comparison between more than two groups, analysis of variance and the Holm-Sidak method) for normalized data or Friedman’s rank sum test (nonparametric method) for the data that did not pass the normality test. For the comparison between more than two groups, analysis of variance and the Holm-Sidak method for multiple pairwise comparisons was used. A value of $p < 0.05$ was considered to be significant.

**Results and Discussion**

Antimicrobial sulfonamides, such as SMX, and the sulfone DDS are important drugs for the treatment of *Pneumocystis carinii* pneumonia, especially in AIDS patients (Goldie et al., 2002). However, in this patient population, these drugs are commonly associated with minor to severe cutaneous drug reactions, which are believed to secondary to their metabolism to reactive arylhydroxylamine and arylnitroso derivatives (Svensson, 2003). Because COX-2 is induced in the presence of various forms of environmental and pathological stress (Maier et al., 1990; Buckman et al., 1998), we hypothesized that the increased frequency of these reactions observed in AIDS patients may be secondary to elevated levels of these reactive metabolites formed as a result of COX-2 induction. As an initial test of this hypothesis, we sought to determine whether COX-2 was capable of generating these reactive metabolites.

Using an adduct-specific ELISA, we found that addition of DDS to an incubation mixture containing COX-2, hematin, EDTA, arachidonic acid, and H$_2$O$_2$ resulted in covalent adduct formation (Fig. 1). Importantly, however, a nonselective inhibitor for COX-1 and COX-2 (INDO) did not attenuate the formation of drug/metabolite-protein adducts. In addition, use of lower concentrations of H$_2$O$_2$ in the incubation mixture did not give rise to detectable covalent adducts. Various controls ruled out nonspecific binding of the primary antisera or secondary antibody as causing artifactual results. We confirmed the catalytic activity of COX-2 in this incubation mixture using reactive oxygen species generation as a positive control, as described under Materials and Methods. There was a 2.3-fold increase in the fluorescence of incubations with COX-2 as compared to incubations without COX-2 (data not shown), confirming the catalytic activity of the enzyme.

Formation of the arylhydroxylamine metabolites of DDS and SMX in the incubation mixture was confirmed via HPLC (Fig. 2). However, removal of the enzyme itself from the incubation gave rise to similar amounts of arylhydroxylamine metabolite (Fig. 2). This observation suggested that some other component in the incubation mixture was resulting in the chemical oxidation of DDS and SMX. Since removal of H$_2$O$_2$ from the incubation mixture prevented the formation of the arylhydroxylamine (data not shown), we suspected that we were observing a chemical oxidation of the arylamines. Indeed, we found that H$_2$O$_2$ alone gave rise to a concentration-dependent oxidation of SMX and DDS (Fig. 3).

Our results indicate that enzymatic oxidation of SMX and DDS by COX-2 is negligible, but that chemical oxidation via H$_2$O$_2$ may occur. These results are consistent with the report of Rubin and Cumette (1989), who demonstrated that H$_2$O$_2$ was able to oxidize procainamide, giving...
rise to an arylhydroxylamine metabolite. Additionally, Goebel et al. (1999) found that the covalent binding of procainamide arising from an incubation mixture almost identical to that used in the present study was markedly attenuated when H₂O₂ was removed from the incubation. However, these investigators found that in addition to H₂O₂, hematin was required to obtain similar levels of covalent binding in the absence of COX-2. In contrast, we did not find hematin to be an essential component for the N-oxidation of SMX or DDS (data not shown).

Taken together, these data suggest that COX-2 is unlikely to play a significant role in the formation of reactive metabolites of sulfonamides. Indeed, we have recently found that the protein haptanogen observed when SMX and DDS are incubated with normal human keratinocytes is not altered by the addition of nonspecific and specific inhibitors of COX (Wurster et al., 2004). In addition, incubation of keratinocytes with proinflammatory cytokines, which results in the induction of COX-2, does not enhance the covalent binding of SMX or DDS in these cells (F. D. Khan, S. Roychowdhury, P. M. Vyas, and C. K. Svensson, unpublished observations). These observations indicate that induction of COX-2 in the presence of environmental or pathological stress is unlikely to play a role in the increased frequency of adverse reactions to sulfonamides in AIDS patients.

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