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

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Running Title: Cyclooxygenase-2 and the bioactivation of dapsone and sulfamethoxazole.

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Abbreviations: CDR – cutaneous drug reactions; CYP450 – cytochromes P450; DCHF - 2’,7’-dichorodihydrofluorescein; DDS – dapsone; DDS-NOH – dapsone hydroxylamine; FMO – flavin monooxygenase; INDO- indomethacin; MPO- myeloperoxidase; SMX – sulfamethoxazole; SMX-NOH – sulfamethoxazole hydroxylamine.

Recommended section assignment: Short communications
Sulfamethoxazole (SMX) and dapsone (DDS) are believed to mediate their adverse effects subsequent to bioactivation to their respective arylhydroxylamine and arylnitroso 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. Utilizing an adduct-specific ELISA assay, we found that the complete enzyme system gave rise to covalent adducts. However, the non-specific 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 H\textsubscript{2}O\textsubscript{2} 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.
INTRODUCTION

Administration of sulfonamide antimicrobials such as sulfamethoxazole (SMX) and the sulfone dapsone (DDS) has been associated in humans with hypersensitivity reactions that include fever, skin eruptions, hepatotoxicity, and blood dyscrasias (Dujovne D et al., 1967; Rieder MJ 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 CK, 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 MJ et al., 1995; Reilly TP et al., 2000; Vyas PM 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 T et al., 2002; Naisbitt DJ et al., 2002; Roychowdhury S et al., 2005). Thus, the bioactivation of these arylamine xenobiotics to their respective arylhydroxylamine metabolites may be the first and most important step in initiation of these reactions.

Previous studies have demonstrated the ability of various oxidizing enzymes, including cytochrome 450 (CYP) 2C9, CYP2E1 and CYP3A4 as well as myeloperoxidase (MPO) to bioactivate arylamines in vitro (Cribb AE et al., 1990; Cribb AE et al., 1995; Mitra AK et al., 1995; Cashman JR et al., 1999; Winter H et al., 2000). Cyclooxygenases (COX), or prostaglandin H synthase, have also been shown to bioactivate heterocyclic amines to their hydroxylamine metabolites (Liu Y and Levy G, 1998). Procainamide, an arylamine antiarrhythmic agent, has also been found to be oxidized to its arylhydroxylamine and arylnitroso metabolites by COX-2 (Goebel C et al., 1999). The N-oxidation of 4-chloroaniline has also been reported to be mediated by COX (Golly I and Hlavica P, 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 (St. Louis, MO) or Fisher Scientific (Chicago, IL). DDS and SMX hydroxylamine metabolites were synthesized as described previously (Vyas PM et al., 2005). Human recombinant COX-2 was obtained from Cayman Chemical (Ann Arbor, MI). Rabbit anti-sera was raised against SMX- and DDS-keyhole limpet hemocyanine conjugates and specificity assessed as described previously (Reilly TP et al., 2000). Goat anti-rabbit antibody conjugated with alkaline phosphatase was purchased from Molecular Probes (Eugene, OR). 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 non-specific binding to the microtiter plate. After 1 h 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 TP 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) were 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. After 1 h, the reaction was terminated by addition of 3 ml of ethyl acetate and the
arylhydroxylamine metabolites determined as described previously (Reilly TP 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 (DCHF, 5 µM), as we have previously described (Vyas PM et al., 2005).

**Determination of H$_2$O$_2$ mediated arylhydroxylamine formation of SMX or DDS via high performance liquid chromatography (HPLC).** SMX or DDS (800 µM) were incubated in Tris-HCl buffer (50 mM, pH 8.00) and ascorbic acid (1mM) with increasing concentration 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 1h, the metabolites formed were extracted and quantified via HPLC.

**Statistical analysis.** Data are presented as mean (SD). Statistical comparisons between two groups were made using either student t-test (parametric method) for normalized data or Friedman’s rank sum test (nonparametric method) for the data which did not pass the normality test. For the comparison between more than two groups, ANOVA 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 SJ et al., 2002). However, in this patient population, these drugs are commonly associated with minor to severe CDRs, which are believed to be secondary to their metabolism to reactive arylhydroxylamine and arylnitroso derivatives (Svensson CK, 2003). As COX-2 is induced in the presence of various forms of environmental and pathological stress (Maier J et al., 1990; Buckman S 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 if COX-2 was capable of generating these reactive metabolites.

Using an adduct specific ELISA assay, we found that addition of DDS to an incubation mixture containing COX-2, hematin, EDTA, arachidonic acid and H₂O₂ resulted in covalent adduct formation (Fig. 1). Importantly, however, a non-selective 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 H₂O₂ in the incubation mixture did not give rise to detectable covalent adducts. Various controls ruled out non-specific 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 in Materials and Methods. There was 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\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{2} may occur. These results are consistent with the report of Rubin and Curnette (Rubin RL and Curnette JT, 1989), who demonstrated that H\textsubscript{2}O\textsubscript{2} was able to oxidize procainamide giving rise to an arylhydroxylamine metabolite. Additionally, Goebel et al (Goebel C 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 H\textsubscript{2}O\textsubscript{2} was removed from the incubation. However, these investigators found that in addition to H\textsubscript{2}O\textsubscript{2}, 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 mediating the formation of reactive metabolites of sulfonamides. Indeed, we have recently found that the protein haptenation observed when SMX and DDS are incubated with normal human keratinocytes is not altered by the addition of non-specific and specific inhibitors of COX (Wurster W et al., 2004). In addition, incubation of keratinocytes with pro-inflammatory cytokines, which results in the induction of COX-2, does not enhance the covalent binding of SMX or DDS in these cells (Khan FD, Roychowdhury S, Vyas PM, and Svensson CK; 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.
REFERENCES:


DMD #6890

FOOTNOTES

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Figure Legends

Figure 1. Bioactivation and subsequent adduct formation of DDS by human recombinant COX-2. DDS (100 µM) was incubated in a 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 (100 µM) for 1 h at 37°C. Controls containing buffer, COX-2 and COX-2+INDO were used to determine the non-specific binding of anti-DDS rabbit sera. Covalent adducts were determined using adduct specific ELISA as described in Materials and Methods. Data presented represent the mean (SD) optical density of 6 replicates. Data were analyzed statistically using ANOVA and Holm-Sidak test for multiple pairwise comparisons. *p<0.05 compared to buffer control, COX-2 and COX-2+INDO. DDS-BSA was used as positive control.

Figure 2. Determination of COX-2 mediated arylhydroxylamine formation of DDS and SMX by HPLC. SMX or DDS (800 µM) were incubated in a mixture containing arachidonic acid (1 mM), hematin (1 µM), EDTA (5 mM), ascorbic acid (1 mM) and H₂O₂ (1 mM) in Tris-HCl buffer (50 mM, pH 8.00), with and without COX-2 (100 units), for 1 h at 37°C. After 1 h, the formed SMX-NOH or DDS-NOH was extracted and quantified via HPLC as described in Materials and Methods. Data presented represent the mean (SD) amount of SMX-NOH formed of 9 replicates of each condition. Data were analyzed statistically using the Student’s t-test, with no differences between incubation conditions observed.

Figure 3. Determination of H₂O₂ mediated arylhydroxylamine formation of DDS and SMX by HPLC. DDS and SMX (800 µM) were incubated with increasing concentrations of H₂O₂ with ascorbic acid (1 mM) in Tris-HCl buffer (50 mM, pH 8.00) for 1 h at 37°C. After 1 h, the formed hydroxylamine metabolites were extracted and quantified via HPLC as described in Materials and Methods. Data presented represent the mean (SD) amount of DDS-NOH and SMX-NOH formed for 9 replicates of each condition.
Figure 1

[Graph showing optical density at 405nm for different treatments: DDS-BSA, Buffer Control, COX-2, COX-2+INDO, COX-2+DDS, COX-2+DDS+INDO. Significant differences marked with asterisks (*).]
Figure 2

Hydroxylamine metabolite formed (ng)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Complete incubation mixture</th>
<th>Incubation mixture without COX-2</th>
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<tbody>
<tr>
<td>SMX</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>DDS</td>
<td>250</td>
<td>250</td>
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</table>
Figure 3

The graph shows the amount of hydroxylamine formed in ng in response to different concentrations of H$_2$O$_2$. The concentrations tested range from 0.01 µM to 10 mM. The graph compares the formation of SMX-NOH (black bars) and DDS-NOH (gray bars) at each concentration level.