Late administration of COX-2 inhibitors minimize hepatic necrosis in chloroform induced liver injury

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Abstract

Our previous studies have described the protective effects of hepatoprotective agents against liver injury elicited by chloroform even when given 24 h after the toxicant, at a time when the liver injury is taking place and rapidly developing. However, the mechanisms involved in this protection remain unknown. The cytoprotective mechanism of these hepatoprotectants such as DMSO, may be due to a dramatic shift in the production of prostaglandins that are responsible for controlling the degree of inflammatory response that can affect blood flow in the liver. In this study, NS-398, a specific COX-2 inhibitor, and indomethacin, a COX-1 and COX-2 inhibitor, were administered 24 h after chloroform dosing to determine their effect on liver injury in Sprague–Dawley rats. The extent of necrosis was evaluated by H&E staining, while injury to hepatocytes was evaluated by measuring plasma levels of alanine transaminase (ALT). Both COX inhibitors, indomethacin and NS-398, prevented an increase in (ALT) at 48 h after initial toxicant insult and attenuated further liver necrosis. No changes in cellular proliferative activity occurred in all the treatment groups, which indicates that protection from the Cyclooxygenase (COX) inhibitors did not have an effect on regeneration of cells at 32 and 48 h. These results indicate COX inhibitors provide a significant protective effect on liver cells against CHCl₃ injury and may provide further insight into therapeutic interventions against hepatotoxins.

Keywords: Sprague–Dawley rats; Cyclooxygenase-1; Cyclooxygenase-2; Hepatotoxicity; Chloroform

1. Introduction

Injury to hepatocytes by a toxicant stimulates the release of a cascade of factors by the cells in the liver. Immediately following injury, formation of arachidonic acid (AA) metabolites in the liver contributes to the initiation of acute inflammation. Cyclooxygenase (COX) enzymes are key inflammatory mediators which convert AA to prostaglandin H₂, the precursor of inflammatory mediators such as prostaglandin E₂ (PGE₂), prostacyclin and thromboxane A₂ (Fu et al., 1990; Hawkey, 1999). Two COX isoforms have been identified (Xie et al., 1991; Kujubu et al., 1991). COX-1 is associated with prostaglandin produc-
tion required for normal physiological organ function and is expressed in a variety of cells and tissues (O’Neill and Ford-Hutchinson, 1993). In contrast, COX-2, is induced at sites of inflammation or other cellular stresses by endotoxin, mitogens, and cytokines (Vane et al., 1994; Gilroy et al., 1998). Overexpression of COX-2 has been observed in various inflammatory diseases, such as rheumatoid arthritis, and ulcerative colitis (Kang et al., 1996; Singer et al., 1998).

The COX pathway may be a major factor in the attenuation of liver injury. Selective inhibitors were used to investigate the contribution of COX isoforms on hepatoprotection. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) inhibits both COX isoforms. In contrast, NS-398 is highly selective for COX-2 (Futaki et al., 1994). The inhibition of COX-2 can block inflammatory responses and protect disruption of the blood–nerve barrier by reducing prostaglandin synthesis (Miyamoto et al., 1999). Selective COX-2 inhibitors have been shown to suppress inflammation in rheumatoid arthritis (Katori et al., 1998). Inhibition of COX with indomethacin and NS-398 blocks the downstream production of prostanooid vasodilatory metabolites. In the liver, endothelial and Kupffer cells are considered the major cellular sources of prostaglandins, while hepatocytes rapidly metabolize COX products (Billiar et al., 1990; Decker, 1991).

In this study, in vivo experiments were conducted to determine if inhibition of COX-2 was involved in the attenuation of liver necrosis after initial treatment with CHCl₃. The COX inhibitors tested in Sprague–Dawley rats were indomethacin, a dual COX-1 and COX-2 inhibitor, and NS-398, a specific COX-2 inhibitor. Nordihydroguaiaretic acid (NDGA), a LOX inhibitor is responsible for another pathway of AA metabolism and was used to compare the effect of a LOX inhibitor with COX inhibitors.

The hepatoprotective effects of COX inhibitors against CHCl₃ in Sprague–Dawley rats were compared with previous studies where DMSO and aminobenzotriazole (ABT) were shown to protect against further liver injury induced by CHCl₃ (Lind and Gandolfi, 1999a,b; Lind et al., 2000). Thus DMSO and ABT were included to give a reference point for the potency of the COX and LOX inhibitors.

Previous studies have shown that DMSO and ABT suppressed the hepatotoxic effect of several different xenobiotic compounds (e.g. chloroform, bromobenzene, halothane) in Sprague–Dawley rats when given 24 h after the chemical exposure, a time when liver necrosis is progressing (Siegers, 1978; Lind and Gandolfi, 1997, 1999a,b; Lind et al., 2000). Chloroform was selected for these subsequent studies as a model compound since it produces a very reproducible, time-dependent liver injury in our rats. As liver injury develops, microcirculation through the narrow sinusoidal channels of the liver is reduced. However, treatment with DMSO and ABT, re-established blood flow in the liver by decreasing the number of activated Kupffer cells (Ito et al., 2000).

Acute chloroform (CHCl₃) administration in Sprague–Dawley rats results in hepatic damage, which peaks at 48 h after dosing. Based on a previous time course of injury study, measurements were selected at 32 and 48 h after CHCl₃ dosing as this is the time during which DMSO begins to attenuate the developing injury (Lind and Gandolfi, 1999a). Initial injury to hepatocytes by a toxicant involves the release of various mediators by activated Kupffer cells including cytokines, reactive oxygen intermediates, and AA metabolites (Laskin, 1990). Evidence of hepatoprotection against chemically induced injury includes decreases in both ALT activity and the extent of hepatic centrilobular necrosis (Lind and Gandolfi, 1999b).

The study provides information for evaluating the role of the COX inhibitors to intervene in the development of liver injury at such a late time in the process of liver injury recovery. These studies may provide potential therapeutic agents for liver injury induced by toxicants.

2. Materials and methods

2.1. Chemicals

Chloroform, dimethyl sulfoxide, indomethacin, NDGA, ABT, and ALT ten reagent were pur-
chased from Sigma-Aldrich (St. Louis, MO). NS-398 (N-[2-cyclohexyloxy]-4-nitrophenyl methane-sulphonamide) was obtained from Cayman Chemical Company (Ann Arbor, MI).

2.2. Animals

Male Sprague–Dawley rats (250–300 g) were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and acclimated to our animal facility for at least 7 days prior to use. The animals were maintained on a 12 h day:12 h night cycle and received Rat/Mouse Diet and water ad libitum. All protocols were approved by the University of Arizona Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals guidelines.

2.3. CHCl 3-induced liver injury and hepatoprotectants

Chloroform was administered to groups of animals (N = 8) via oral gavage at 0.75 ml/kg CHCl 3, 20% in corn oil. The hepatoprotective agents (DMSO and ABT) and the inhibitors (indomethacin, NS-398 and NDGA) were administered via intraperitoneal (i.p.) injection 24 h after CHCl 3 dosing. As reported previously, the DMSO treatment was 2 ml/kg DMSO in 50% saline the ABT dose was 30 mg/kg in saline (Lind and Gandolfi, 1999a,b; Lind et al., 2000). The doses of the COX and LOX inhibitors were selected from literature (Katori et al., 1998; Tsugawa et al., 1999; Bandeira-Melo et al., 2000; Mikuni et al., 1998; Miyamoto et al., 1999). Preliminary studies (unpublished data) were performed to determine the optimal dose of NS-398, indomethacin, and NDGA for effective protection against CHCl 3. The selected doses of indomethacin and NS-398 inhibited plasma PGE 2 production up to 24 h after dosing by 83 and 57%, respectively, while NDGA inhibited plasma LTB 4 production up to 8 h after dosing by 64%. At these dose levels no toxic side effects of the COX or LOX inhibitors were seen and there are no reported effects in the literature.

For the eicosanoid inhibition studies, each group (N = 8) received either 10 mg/kg NDGA in saline, 20 mg/kg indomethacin or 1 mg/kg NS-398 (dissolved in ethanol; diluted with saline). Indomethacin was dissolved in 0.1 N NaOH buffered with Tris and neutralized with 0.1 N HCl as previously described (Bandeira-Melo et al., 2000). The CHCl 3 only–‘saline’ group (N = 8) received saline, i.p. in place of the hepatoprotective agent at 24 h after CHCl 3.

2.4. Sample collection

Previous studies indicated that ALT values for the treatment groups began to diverge approximately 8 h after DMSO or ABT administration (Lind and Gandolfi, 1999a). Therefore, sample collection times for assessing the status of liver injury were selected at 32 and 48 h after CHCl 3 dosing. The rats were killed with a lethal (200 mg/kg) dose of i.p. pentobarbital. Cardiac blood was obtained while under deep anesthesia at 32 and 48 h after CHCl 3 dosing and centrifuged to obtain plasma. Plasma samples were immediately analyzed for ALT activity. Livers were removed and a section was cut longitudinally into strips and placed in neutral buffered formalin. Blood and liver tissue was also obtained from a group of untreated control animals. ALT activity was expressed as Units/liter (U/l).

2.5. Histopathology

All liver sections were fixed in formalin solution (10% neutral buffered, Sigma). These were later embedded in paraffin and stained with hematoxylin and eosin (H&E). The extents of liver necrosis produced by CHCl 3 and subsequent protection by the hepatoprotectants were examined at 32 and 48 h. The morphologic changes produced in the liver by CHCl 3 were examined during the time course of the experiment. Necrosis scoring was performed using the SIMPLIPC1 software (C Imaging, Inc.). Digital images were acquired with a SPOT Jr camera mounted on a Nikon E400 microscope using a 10x objective. The score was determined by dividing the measured necrotic area by the total area of the field.
2.6. Mitotic index

The proportion of cells undergoing mitosis was determined with H&E slides. Cells in the prophase, metaphase, anaphase or telophase of the cell cycle were identified manually. The mitotic index was assayed as the number of mitoses observed per ten high power fields chosen at random as previously described (Theocharis et al., 2001). On each slide, ten fields were viewed by light microscopy (400 x) and 20 cells were counted (i.e. 200 hepatocytes per slide). In each pre-determined-field a 'zig-zag' pattern was used to select the cells. The mitotic index was determined as the percentage of the 200 hepatocytes in various phases of the cell cycle.

3. Statistical analysis

All data were analyzed using STATAQUEST version 4.0 for Windows (College Station, TX). Test of significance of differences between treated and saline-treated groups were calculated using the nonparametric Kruskal–Wallis one way analysis of variance and using Wilcoxon rank-sum tests Bonferroni-corrected for multiple comparisons as post hoc significance tests. Data are expressed as mean ± S.E.M. for the animals in each treatment group. P < 0.05 was considered to be statistically significant.

4. Results

To determine the hepatoprotective effects of COX inhibitors, Sprague–Dawley rats received CHCl3 p.o. and 24 h later received DMSO, ABT, NDGA, indomethacin, NS-398 or saline i.p. Based on previous studies (Lind and Gandolfi, 1999b; Lind et al., 2000) the dose of chloroform (0.75 ml/kg) was selected to achieve maximal ALT levels at 48 h without causing death in Sprague–Dawley rats. In this study 98% of the animals survived. The 'saline' treated rats (CHCl3 treated but no inhibitor) as well as the control (untreated rats) were killed at 48 h.

As previously reported in a more detailed time course of CHCl3-induced liver injury study (Lind et al., 2000), at the 32 h time point, the ALT levels and necrosis were not significantly different. Therefore, this time point was not used in the hepatoprotection studies. However, for the mitotic index evaluation, the 32 h time point was included since by 48 h the hepatoprotection was evident and all relevant cell proliferation should have occurred prior to 48 h.

4.1. Hepatoprotection against CHCl3 liver toxicity—ALT levels

ALT levels were measured in the plasma of all rats at 48 h after CHCl3 (Fig. 1). CHCl3-induced liver injury in rats resulted in maximal plasma enzymes (ALT) and peak hepatic necrosis at 48 h. For comparative purposes, ALT levels were measured in rats given DMSO or ABT at 24 h after chloroform. The extent and location of hepatoprotection by DMSO and ABT was identical to that previously reported (Lind and Gandolfi, 1999b; Lind et al., 2000). Both DMSO and ABT, significantly reduced the ALT levels by 86% and 84%, respectively (data not shown). Of the three eicosanoid inhibitors tested, both the nonselective COX inhibitor, indomethacin, and NS-398, the COX-2 specific inhibitor displayed significant

![Graph showing ALT levels after CHCl3](image-url)
protection against further liver injury. Indomethacin and NS-398 reduced ALT levels by 50.6% and 49.0%, respectively, when measured at 48 h after CHCl₃ ingestion. In contrast, NDGA, a LOX inhibitor did not show any protective effects against CHCl₃ induced toxicity.

4.2. Hepatoprotection against CHCl₃ liver toxicity—histopathology

To confirm that the reduction in ALT reflected a reduction in liver damage, liver sections stained by H&E were examined to determine the extent of inflammation, the number of necrotic cells, and hepatocyte proliferation. The extent of liver necrosis as measured by histopathological analysis confirmed the plasma enzyme levels. Substantial necrosis (48%±7.11) was noted in the liver with peak ALT levels of 3287±524 at 48 h after CHCl₃. Both indomethacin and NS-398 were more effective at protecting against further liver damage than the LOX inhibitor, NDGA. Extensive hepatic necrosis was observed at 48 h in the CHCl₃ treated rats followed by saline treatment (Fig. 2b). In these rats, the necrotic areas included ballooned hepatocytes due to macrovesicular steatosis and the extent of necrosis bridged to other centrilobular vein. Additionally, mesenchymal cells accumulated in the centrilobular area and infiltration of neutrophils was clearly visible. During peak necrosis, the necrosis score for CHCl₃-treated rats was 48%±7.11 (Fig. 3). Substantial areas of centrilobular injury were present in the rats receiving lipooxygenase inhibitor, NDGA as the protective agent (37%±5.9; Fig. 2c). However, the rats, treated with indomethacin or NS-398 after the CHCl₃ dosing, showed a substantial decrease in necrosis score to 18.2%±4.9 and 12.9%±1.9, respectively (Fig. 3). The injury was limited to just a few cells around the central vein (Fig. 2c and d).

4.3. Liver regeneration

To examine the possibility that the hepatoprotective effects of COX inhibitors may have resulted from an increase in acceleration of recovery through cell division, the effects of COX inhibitors on the mitotic index of CHCl₃-induced liver cells were examined. For the CHCl₃-induced rats, there were no significant changes in the mitotic index at 32 or 48 h after initial toxicant dose (Table 1). Neither DMSO nor ABT nor the eicosanoid inhibitors had an effect on hepatocyte regeneration when given at 24 h after CHCl₃. This results suggest that hepatoprotection by these agents did not occur by accelerated cell division.

5. Discussion

The study was designed to evaluate whether inhibition of COX activity would have a hepatoprotective effect against CHCl₃-induced liver injury. The principle findings of this study show that administration of COX inhibitors, indomethacin or NS-398, at 24 h after initial CHCl₃ dose, was beneficial in this model in protecting against progressing liver injury.

The administration of CHCl₃ in rats caused severe liver injury, evident through both histopathology and increased ALT activities. The mechanism responsible for CHCl₃ induced hepatic toxicity is believed to be mediated by reactive intermediate metabolites formed in the course of CHCl₃ biotransformation in the liver (Pohl et al., 1980; Hewitt et al., 1980). The development of hepatic necrosis in rats becomes prominent as early as 10 h after administration of CHCl₃ and peaks at 48 h (Lind et al., 2000). After 48 h, a hepatic regenerative process occurs, as tissue repair is a biological response that accompanies chemically-induced liver injury.

In response to liver injury, inflammation reactions are activated and inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and AA metabolites, such as thromboxane A₂, prostaglandin I₂, PG₂, and leukotrienes are released (Ljungman et al., 1991). AA is metabolized to bioactive eicosanoids by three major pathways, COX, LOX, and NADPH-dependent cytochrome P-450 monooxygenase enzymatic pathways (Smith, 1992). The formation of these eicosanoids in the liver contributes to the initiation of the acute phase response to inflammation. COX enzymes catalyze the conversion of AA to active prosta-
PGE$_2$ is a major product of COX activity and contributes to inflammation by causing vasodilation and potentiating the effects of mediators such as bradykinin and IL-1 (Davies et al., 1984). In addition, AA metabolites produced by COX play a role in apoptotic cell death (Iida et al., 1998).

COX-1 is involved in physiological prostaglandin formation and is expressed in most types of cells and tissues (O’Neill and Ford-Hutchinson, 1993). COX-2 is induced and activated at sites of inflammation by endotoxin, mitogens, and cytokines (Vane et al., 1994; Gilroy et al., 1998). Thus, the inhibition of COX enzyme activities by our hepatoprotective agents is essential and may have dramatic effects in protecting against liver injury and preserving the liver structure.

We examined the effects of COX inhibitors on hepatotoxicity using indomethacin and NS-398. Inhibition of COX with indomethacin and NS-398 block the downstream production of vasodilator prostanoid metabolites (Poyser, 1999). Indomethacin has been shown to inhibit the conversion of AA to PGE$_2$ and thus prevents exacerbation of the existing injury (Rufer et al., 1982). Indomethacin was effective in our model by suppressing both ALT levels and further liver necrosis when measured at a time when peak necrosis should occur.

In this study, we employed NS-398, to investigate the role of COX-2 in liver injury. NS-398 was reported to attenuate the increase in tumor necrosis factor-$\alpha$, a cytokine involved in inflammation (Araki et al., 2001). The overexpression of inducible COX-2 has been associated with vascular inflammation and cellular proliferation. In the present study, NS-398 was able to reduce ALT levels and prevent further liver necrosis, when given 24 h after initial CHCl$_3$ dose. The reduction in ALT levels may again be a result of reduced PGE$_2$. A reduction in prostaglandin synthesis is

<table>
<thead>
<tr>
<th>Treatment</th>
<th>32 h after CHCl$_3$</th>
<th>48 h after CHCl$_3$</th>
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<tbody>
<tr>
<td>Control</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Saline</td>
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<td>0.10 ± 0.01</td>
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<tr>
<td>NS-398</td>
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<td>0.09 ± 0.01</td>
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<tr>
<td>Indomethacin</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.01</td>
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<tr>
<td>NDGA</td>
<td>0.09 ± 0.03</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>ABT</td>
<td>0.07 ± 0.01</td>
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There were no statistically differences between treatment groups. The amount of cells undergoing mitotic activity did not change. Data is shown as mean ± S.E.M. ($N=8$).

### Table 1
Mitotic indices (mitotis/10HPF) of liver regeneration in CHCl$_3$-treated rats examined at different time points post-toxin administration.

Fig. 2. The effects of late administration of COX inhibitors on progressing liver necrosis. (a) Rat liver showing normal histology, (b) CHCl$_3$-treated rat liver, at 48 h post toxin administration. Extensive centrilobular necrosis was observed in these liver section. Large cells surrounding the necrotic zone are hepatocytes ballooned due to macrovesicular steatosis. Extent of necrosis bridges to other centrilobular veins. (c) Late administration of specific COX-2 inhibitor, NS-398, necrosis is limited around centrilobular vein and does not extend to another central vein. (d) Late administration of indomethacin, a COX-1 and COX-2, liver necrosis was limited to cells around the centrilobular vein. (e) Liver of rat given NDGA, 24 h after CHCl$_3$ dosing, necrosis extend to another central vein (H&E, magnification $\times$ 40).

Fig. 3. Assessment of hepatic necrosis in rats administered CHCl$_3$ then treated with COX inhibitors. Results represent area of necrosis over total area at 10 × . Late administration of COX inhibitors induced a significant decrease in liver necrosis compared with rats treated with saline only (*, indicates $P < 0.05$ vs. saline group). There were no statistically differences between NDGA treated group and saline group at $P < 0.05$. Data is shown as mean ± S.E.M. ($N=8$).
known to protect the blood–nerve barrier, which is disrupted during liver injury (Miyamoto et al., 1999). NS-398 can also have an effect on cell proliferation. In a previous report, NS-398 treatment resulted in suppressed cell proliferation by inducing a G0/G1 cell-cycle arrest (Nakanishi et al., 2001). Since there were no changes in the mitotic index in our hepatoprotection model, we are not sure if NS-398 had an effect on cell proliferation in our system.

Although NDGA was able to alleviate Fe-NTA-mediated nephro- and hepatotoxicity and tumor promotion in a prior study (Ansar et al., 1999), the concentration of NDGA used in this study was not sufficient to attenuate liver injury at the 48 h time point. It is possible that NDGA participates in mediating the reduction of LTβ4 at time points earlier than we tested. NDGA inhibits LOX as well as monooxygenase activity and provides a possible mechanism for the protective role of NDGA against the onset of carcinogenesis, which is induced by the chemicals that are metabolized by the cytochrome P-450-dependent enzyme system.

It is important to determine if there are changes in cellular regeneration rates in the liver following injury and therapeutic treatment. The mitotic index was used to measure changes in cellular growth rate. At the time of peak ALT levels and maximal necrosis, CHCl₃ alone did not alter the mitotic index. None of the hepatoprotective agents (DMSO, ABT, indomethacin, NS-398) caused an increase in mitotic index either at the 48 h time point or earlier (32 h) when increased mitosis would need to be present if proliferation the mechanism of protection. Thus the protective effect must result from limiting the initial injury rather than replacing damaged cells.

In conclusion, NS-398 and indomethacin suppressed ALT levels and necrosis in response to CHCl₃-induced liver injury, at a time when liver necrosis is occurring and progressing. The prevention is the result of interfering with the lesion and not a stimulation of cell regeneration. Therefore, our study may provide insight into therapeutic use of COX inhibitors for toxicant induced liver injury at such a late time.

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