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Drug and Chemical Toxicology, 2013; 36(1): 1–8
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ISSN 0148-0545 print/ISSN 1525-6014 online
DOI: 10.3109/01480545.2011.642380

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RESEARCH ARTICLE

Celecoxib, a selective cyclooxygenase-2 inhibitor, lowers plasma cholesterol and attenuates hepatic lipid peroxidation during carbon-tetrachloride–associated hepatotoxicity in rats

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Abstract

Cyclooxygenase-2 (COX-2) expression and prostaglandin production are suggested to play important, complex roles in the pathogenesis of various liver diseases. Studies on the effects of COX-2 inhibitors on the progression of liver fibrosis present controversial results, and the proposed therapeutic potential of these agents in chronic liver disease is predicated largely on their effectiveness in modulating hepatic stellate cell activation *in vitro*. This study investigated the modulatory effect of celecoxib, a selective COX-2 inhibitor, in CCl_4-mediated hepatotoxicity in rats. Thirty Wistar albino rats, weighing 120–180 g, were assigned into five groups of 6 rats/group. Groups 1 and 2 received saline (10 mL/kg) and CCl_4 (80 mg/kg), respectively. Group 3 was given celecoxib (5.7 mg/kg), whereas groups 4 and 5 were pretreated with 2.9 and 5.7 mg/kg/day of celecoxib, respectively, 1 hour before CCl_4 treatment. Plasma aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activities increased significantly by 118.5, 150.0, and 51.3%, respectively, with an accompanying decrease ($P < 0.05$) in total protein and albumin after CCl_4 treatment. Hepatotoxicity was associated with a significant increase in plasma cholesterol, hepatic lipid peroxidation (LPO), and severe hepatic necrosis with marked fatty and cellular (i.e., mononuclear cells) infiltration. Although celecoxib neither reduced CCl_4-induced increases in marker enzymes of hepatotoxicity nor significantly attenuated hepatic necrosis, it, however, was effective in reducing elevated cholesterol by 16.5 and 20.8% and LPO by 12.9 and 35.5% at 2.9 and 5.7 mg/kg, respectively. Data suggest that COX-2 inhibitors may be effective in controlling hypercholesterolemia and peroxidative changes associated with liver injury.

Keywords: Hepatotoxicity, carbon tetrachloride, cyclooxygenase-2 inhibition, celecoxib

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Introduction

The crucial role of the cyclooxygenase (COX)-prostanoid pathway in inflammation, carcinogenesis, hemodynamics, and renal function is well recognized. However, accumulating data also indicate that the COX-2-prostanoid pathway may be associated with various liver diseases (Hu, 2003). The COX enzyme is known to play an important role in arachidonate metabolism, where it catalyzes the first step in the biosynthesis of various prostaglandins (PGs) and thromboxanes (TXs) (Xie et al., 1991; DeWitt, 1991). Two major isoforms of this enzyme have been identified: the constitutively expressed COX-1, which is involved in the production of PGs responsible for homeostatic or "house-keeping" function, and the inducible COX-2, which produces inflammatory PGs in response to a variety of stimuli (Pilbeam et al., 1993; Feng et al., 1995; Kujubu et al., 1991).

Evidence in the literature suggests the role of COX-2 in several signaling pathways in hepatic stellate cells (HSCs). COX-2 expression has been demonstrated in

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(Received 26 August 2011; revised 25 October 2011; accepted 15 November 2011)

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serum-starved HSCs *in vitro*, and this expression was further upregulated by cytokines (Gallois et al., 1998; Mallat et al., 1998). HSC proliferation and migration stimulated by platelet-derived growth factor have also been shown to be associated with COX-2 induction and increased PGE_2 production (Mallat et al., 1998; Failli et al., 2000). Both experimental and clinical data from various studies suggest that increased COX-2 expression and PG production may contribute to liver damage. There are strong evidences that the induction of COX-2 is involved in hepatic inflammatory response in alcoholic and nonalcoholic liver disease (Nanji et al., 1997; Yu et al., 2006). COX-2 induction has been detected in Kupffer cells (Nanji et al., 1997), hepatocytes (Han et al., 2008; Yu et al., 2007), and stellate cells (Gallois et al., 1998). It is now recognized that COX-2-mediated prostanoid generation is involved in early inflammatory response (Deng et al., 2006; Li and Matsumura, 2008). In this process, arachidonic acid is converted into cyclic endoperoxides (mainly PGG_2 and PGH_2), from which prostanoids, including PGs, TXs, and prostacyclins, are synthesized (Smith and Dewitt, 1996; Feng et al., 1995). COX-2 ultimately elicits the onset of inflammation mainly through the production of proinflammatory prostanoids, such as PGE_2 and TXB_2 (Hinson et al., 1996; Nanji et al., 1997). Further, Nunez et al. (2004) reported on COX-2 overexpression in the liver tissue of patients with chronic hepatitis C virus (HCV) infection, observing a positive correlation with the fibrotic stage of HCV-induced liver disease. The intrahepatic expression of COX-2 in these HCV-infected patients was functional, as demonstrated by the parallel increase in PGE_2 levels detected in their liver extracts. Jeong et al. (2010) also demonstrated that COX-2 expression was significantly higher in cirrhosis than in chronic hepatitis, as well as in advanced fibrosis among all tissues, suggesting that COX-2 is involved in the progression of cirrhosis and fibrosis. Similarly, key proinflammatory and -fibrogenic factors involved in the pathogenesis of liver disease have been identified (Friedman, 2000; Bataller and Brenner, 2005; Jaeschke, 2006; Jou et al., 2008). There is also evidence that cholesterol-derived products are capable of inducing these proinflammatory and -fibrogenic mechanisms in liver cells (Ferre et al., 2009). In addition, the existence of a close association between liver disease and the prevalence of metabolic disorders, such as hypercholesterolemia and other forms of dyslipidemia, have been suggested in epidemiological studies (Must et al., 1999). Some investigators, though, observed that hyperlipidemia may not play a causal role in liver injury; they suggested, however, that it may affect the severity of tissue damage (Jou et al., 2008). For example, studies have shown that excess accumulation of cholesterol and other lipids in the cytosol of hepatocytes (which *per se* does not appear to impair liver function) significantly increased the vulnerability of the liver to the deleterious effects of cytokines, oxidative agents, and viral infections, thus predisposing this organ to inflammation and advanced fibrosis (Farrell and Larter, 2006; McIntyre et al., 1999). The potential therapeutic role of COX-2 inhibition in chronic liver disease relies partly on its effectiveness in modulating HSC activation (Cheng et al., 2003) and inhibiting proliferation of human hepatoma cells (Hu et al., 2003) *in vitro*. Although the effects of treatment with COX-2 inhibitors on the progression of liver fibrosis have been investigated in various experimental models, results from these studies, however, are controversial. Though some investigators reported exacerbation (Hui et al., 2006; Reilly et al., 2001; Yu et al., 2008), others observed amelioration (Yamamoto et al., 2003; Denda et al., 1997; Tu et al., 2007; Planaguma et al., 2005) in fibrogenesis after COX-2 treatment. Overexpression of COX-2 messenger RNA has been demonstrated in rats with CCl_4-induced cirrhosis, and indomethacin (a nonselective inhibitor of COX) decreased the hyperresponse to vasoconstrictors and increased resistance to portal flow—the primary factor in the pathophysiology of portal hypertension in cirrhotic livers (Graupera et al., 2003). With reference to recent evidence pointing to a potential therapeutic role of modulating the COX-prostanoid pathway in liver disease, we evaluated the possible protective benefit of celecoxib, a selective COX-2 inhibitor, in a CCl_4 model of liver injury in rats in this present study. This was with a view to ascertaining or identifying this pathway and, in particular, the COX-2 enzyme as a potential therapeutic target in the management of xenobiotic or chemically mediated liver injury.

Methods

Drugs and chemicals

Celecoxib (Celebrex®) was obtained from Pfizer Inc. (Freiburg, Germany). Thiobarbituric acid (TBA) and 5′,5′-dithiobis-2-nitrobenzoate (Ellman's reagent) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). Reduced glutathione (GSH), metaphosphoric acid, and trichloroacetic acid (TCA) were purchased from J.L. Baker (Phillipsburg, New Jersey, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total cholesterol (TC), and triglyceride (TG) assay kits were obtained from Randox Laboratory (Crumlin, UK). All other chemicals and reagents used were of analytical grade.

Animals

Male Wistar albino rats, obtained from a commercial private colony in Ibadan, Nigeria, were housed at ambient temperature and humidity with a 12-hour light-dark schedule within the experimental animal-handling facility of the Department of Pharmacology, Olabisi Onabanjo University (Ago-Iwoye, Nigeria). They were placed on a rat pelleted diet (Bendel Feed & Flour Mill Ltd., Ewu, Nigeria) and water *ad libitum* during the period of acclimatization and throughout the period of the experiment. Study was in accord with established guidelines for the care and use of laboratory animals in biomedical research.

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Experimental design and treatment

A total of 30 rats (weighing 120–200 g) were divided into five groups of 6 rats per group. Hepatotoxicity was induced by the subacute administration of CCl_4 (80 mg/kg/day) dissolved in corn oil (0.05 mL CCl_4/ml). The various groups were treated as follows: group 1: physiological saline (10 mL/kg/day); group 2: CCl_4 (80 mg/kg/day); group 3: celecoxib (2.9 mg/kg/day) plus CCl_4 (80 mg/kg/day); group 4: celecoxib (5.7 mg/kg/day) plus CCl_4 (80 mg/kg/day); and group 5: celecoxib (5.7 mg/kg/day).

Rats in groups 3 and 4 were pretreated with celecoxib 1 hour before CCl_4 administration. All treatment was given via the oral route for a period of 11 days, starting with 5 days of administration followed by 2 days of rest and then with a continuous administration for 4 days (Bruckner et al., 1986). Celecoxib was administered at the therapeutic and twice the therapeutic doses and calculated based on 70 kg body weight for a physiological man (therapeutic dosing of celecoxib is 200 mg/70 kg body weight).

Necropsy

Rats were sacrificed by cervical dislocation 24 hours after the last treatment, and blood was collected by cardiac puncture into lithium heparin bottles. Plasma was separated after centrifugation at 4,200 rpm at room temperature for 5 minutes. The liver was carefully excised, cleared of adhering tissues, and weighed. Weight was recorded in grams and expressed as g/kg body weight. A small portion of the excised liver was fixed in 10% formaldehyde and subsequently prepared for histology. The remaining portion of the excised liver was weighed and homogenized in four volumes of 100 mM of phosphate buffer (pH 7.4). The plasma and liver homogenate obtained from each animal were then analyzed to assess liver function and other biochemical parameters.

Biochemical analysis

Liver function was assessed by measuring the activities of AST, ALT, and ALP in plasma. AST and ALT activities were determined according to the principle described by Reitman and Frankel (1957), whereas ALP activity was carried out according to the method described by Roy (1970). To assess the synthetic function of the liver, total protein and albumin concentrations were carried out according to the principle based on Biuret reaction (Gornall et al., 1949) and bromocresol green reaction (Doumas et al., 1971), respectively. GSH level was estimated at 412 nm, following the method of Beutler et al. (1963). Lipid peroxidation (LPO) was estimated spectrophotometrically by the thiobarbituric acid reactive substance method, as described by Varshney and Kale (1990), and expressed in terms of malondialdehyde (MDA) formed per mg of protein. TC and TG concentrations were estimated by following the principle described by Trinder (1969) using commercial kits obtained from Randox Laboratories Ltd. (Crumlin, UK).

Statistical analysis

Data presented are mean ± standard error of the mean (SEM) and were analyzed using Statistical Package for Social Sciences (SPSS) software for windows (SPSS, Inc., Chicago, Illinois, USA). Differences between groups were determined by one-way analysis of variance, and post-hoc testing was performed for intergroup comparisons using the least significant difference (LSD) (Levine, 1991). A P-value <0.05 was considered significant.

Results

Marker enzyme for liver function

Results of liver function tests are shown in Table 1. ALP activity increased significantly ($P < 0.05$) by 51.3%, whereas the activities of both AST and ALT increased significantly ($P < 0.001$) by 118.5 and 150.0%, respectively, in the CCl_4-treated rats. Celecoxib, at therapeutic (2.86 mg/kg) and twice the therapeutic (5.72 mg kg) doses, did not attenuate CCl_4-induced increase in ALP, AST, and ALT activities and impairment of hepatic function in these animals. Administration of celecoxib (5.72 mg/kg) alone did not alter hepatic function, as revealed by the comparable activities of the plasma-marker enzymes in rats treated with the drug with those of the control.

Total protein and albumin

The elevated increase in marker enzyme of liver function induced by CCl_4 was associated with significant decreases in albumin ($P < 0.001$) and total protein ($P < 0.05$) concentrations (Figure 1). Celecoxib, at the therapeutic (2.86 mg/kg) dose, significantly ($P < 0.001$) prevented CCl_4-associated increase in these serum

Table 1. Effect of celecoxib on plasma activities of AST, ALT, and ALP in normal and CCl_4-treated rats.

Treatment	AST (mg/dL)	ALP (mg/dL)	ALT (mg/dL)
Control (NS, 10 mL/kg)	57.8 ± 8.24	44.0 ± 2.0	30.6 ± 3.7
CXB (5.7 mg/kg)	57.6 ± 1.4 (0.3)[a]	44.9 ± 6.1 (–1.2)[a]	27.5 ± 4.0 (10.1)[a]
CCl_4 (80 mg/kg)	126.2 ± 4.2* (–118.5)[a]	67.2 ± 6.2** (–51.3)[a]	76.5 ± 3.7* (–150.0)[a]
CCl_4+CXB (2.9 mg/kg)	128.5 ± 2.9 (–1.8)[b]	56.1 ± 6.5 (16.5)[b]	83.8 ± 2.0 (–9.5)[b]
CCl_4+CXB (5.7 mg/kg)	126.0 ± 5.1 (0.2)[b]	61.1 ± 3.31 (9.1)[b]	84.0 ± 4.2 (–9.8)[b]

Results are expressed as mean ± SEM. Values in parenthesis represent % change; (–) = increase; (+) = decrease.
[a]% change relative to control group.
[b]% change relative to CCl_4.
*$P < 0.001$, when compared with the control group; **$P < 0.05$, when compared with the control group.
CXB, celecoxib; NS, normal saline.

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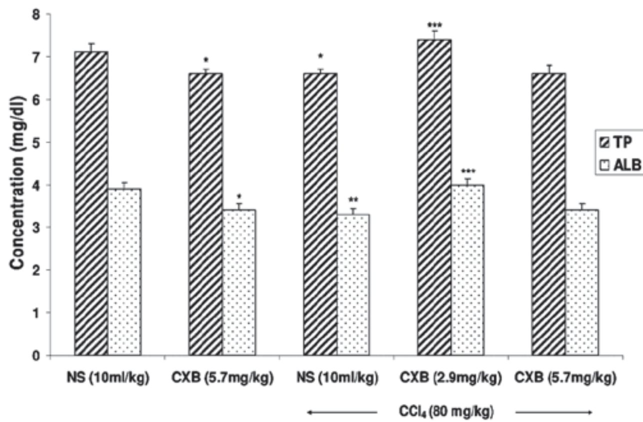

Figure 1. Effect of celecoxib on plasma total protein and albumin in normal and CCl_4-treated rats. Results are expressed as mean ± SEM. *$P<0.05$ and **$P<0.001$, when compared with control; ***$P<0.001$, when compared with CCl_4. CXB, celecoxib; NS, normal saline; TP, total protein; ALB, albumin.

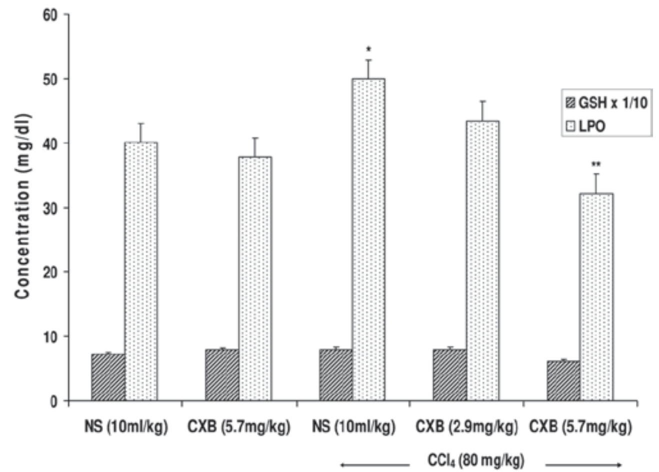

Figure 2. Effect of celecoxib on hepatic glutathione and LPO in normal and CCl_4-treated rats. Results are expressed as mean ± SEM. *$P<0.05$ and **$P<0.001$, when compared with control and CCl_4, respectively. CXB, celecoxib; NS, normal saline.

Table 2. Effect of celecoxib on plasma total cholesterol and triglyceride in normal and CCl_4 treated rats.

Treatment	TC (mg/dL)	TG (mg/dL)
Control (NS, 10 mL/kg)	107.3 ± 6.6	78.7 ± 11.3
CXB (5.7 mg/kg)	105.5 ± 7.4 (1.7)[a]	77.2 ± 15.3 (2.00)[a]
CCl_4 (80 mg/kg)	130.4 ± 5.0* (–21.6)[a]	85.3 ± 3.4 (–8.3)[a]
CCl_4+CXB (2.9 mg/kg)	108.9 ± 3.3** (16.5)[b]	87.3 ± 15.6 (4.7)[b]
CCl_4+CXB (5.7 mg/kg)	103.3 ± 5.2** (20.8)[b]	106.9 ± 17.6 (–25.4)[b]

Results are expressed as mean ± SEM. Values in parentheses represent % change; (–) = increase, (+) = decrease.
[a]% change relative to control group.
[b]% change relative to CCl_4.
*$P<0.05$, when compared with control group; **$P<0.05$, when compared with CCl_4.
CXB, celecoxib; NS, normal saline.

proteins in the pretreated animals, whereas it did not provide any ameliorative effect at double the therapeutic dose (i.e., 5.72 mg/kg). When administered alone, celecoxib (5.72 mg/kg) also significantly ($P<0.05$) reduced serum total protein and albumin levels.

TC and TG

Table 2 shows the effect of various treatments on plasma levels of TC and TGs. Elevated plasma cholesterol in the CCl_4-treated significantly ($P<0.05$) reduced by 16.5 and 20.8% after daily treatment with celecoxib at 2.86 and 5.72 mg/kg, respectively. Though celecoxib, when administered alone, did not affect plasma concentration of TG and TC in the normal or saline control rats, it also did not produce any significant change in the concentration of the former in the CCl_4-treated rats, when compared with rats that received CCl_4 alone.

LPO and reduced glutathione (GSH)

Celecoxib decreased LPO by 12.9 ($P>0.05$) and 35.5% ($P<0.001$) at 2.86- and 5.72-mg/kg doses, respectively, in the CCl_4-treated rats (Figure 2). MDA levels in rats treated with celecoxib (5.72 mg/kg) alone were comparable with those of the saline control, showing that this drug is not associated with the induction of LPO. GSH concentration, on the other hand, was not significantly altered and was similar in all the treatment groups.

Histopathology

Representative photomicrographs of liver histology of rats in all treatment groups are presented in Figure 3. Rat livers treated with CCl_4 were characterized by severe hepatic centrilobular necrosis and cellular infiltration by mononuclear cells with fatty infiltration. There was no visible lesion associated with celecoxib (5.72 mg/kg) when administered alone. This drug also did not significantly attenuate the morphological damage associated with CCl_4 toxicity in this study.

Discussion

Although the expression of COX-2 under rapid response to proinflammatory challenge is almost restricted to the nonhepatocyte cell population in the adult liver, it also recognized that the hepatocytes under chronic proinflammatory conditions express this isoenzyme, and the contribution of the increased synthesis of prostanoids to liver pathology has recently become a subject of intense discussion (Cervello and Montalto, 2006; Giannitrapani et al., 2009; Martín-Sanz et al., 2010). It is established that liver cirrhosis induced by CCl_4 is associated with COX-2 overexpression (Graupera et al., 2003). Our result suggests that COX-2 inhibition may not attenuate liver injury associated with CCl_4 intoxication. Celecoxib, a selective COX-2 inhibitor, did not ameliorate the marked increase in plasma ALTs and ASTs as well as ALP characterizing CCl_4-induced hepatotoxicity in this study. Similarly, damage to the liver architecture of CCl_4-intoxicated rats, which manifested as severe centrilobular hepatic necrosis with cellular (i.e., mononuclear cells) and fatty infiltration, was also not resolved by celecoxib administration.

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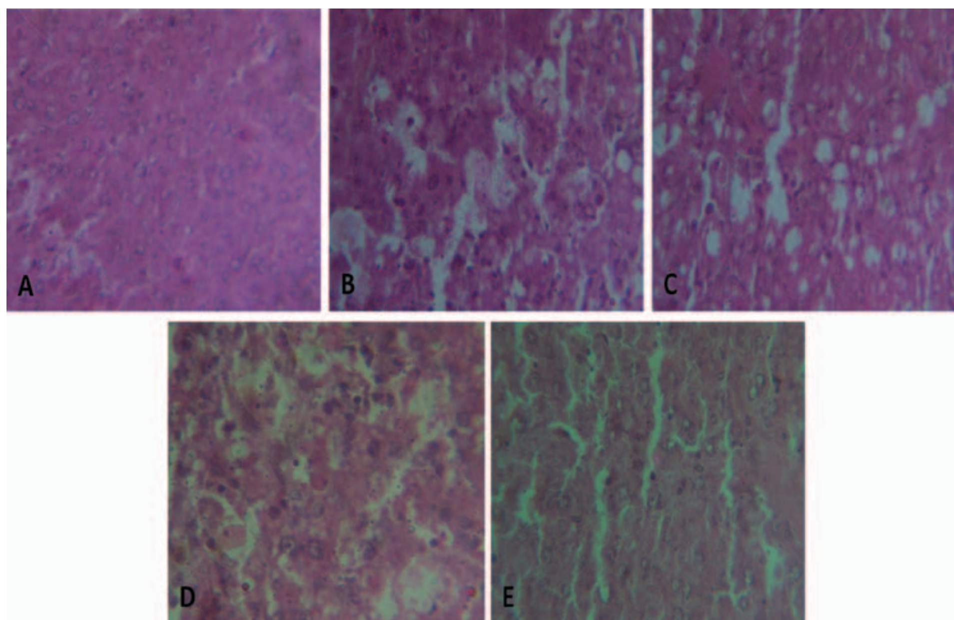

Figure 3. Liver section (×400) of rat treated with (**A**) normal saline (10 mL/kg) without any visible lesion. (**B**) CCl_4 (80 mg/kg) showing very severe centrilobular hepatic necrosis and cellular infiltration by mononuclear cells with marked fatty infiltration. (**C**) CCl_4+CXB (2.9 mg/kg) showing moderate centrilobular hepatic necrosis and cellular infiltration by mononuclear cells with marked fatty infiltration. (**D**) CCl_4+CXB (5.7 mg/kg) showing very severe centrilobular hepatic necrosis and cellular infiltration by mononuclear cells with marked fatty infiltration. (**E**) CXB (5.7 mg/kg) without any visible lesion.

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Our findings could not confirm the reports of previous studies, where COX-2 inhibition has been linked with hepatoprotection in experimental studies. Horrillo et al. (2007) reported on the protective effect of SC-236, a selective COX-2 inhibitor, against liver inflammation and fibrosis and proposed that the COX pathway of the arachidonic acid cascade might represent a potential target for therapy. Similar observations have also been reported by some other investigators (Yamamoto et al., 2003; Denda et al., 1997; Tu et al., 2007; Planaguma et al., 2005). There are other studies, however, that have reported exacerbation in fibrogenesis after COX-2 inhibition (Hui et al., 2006; Reilly et al., 2001; Yu et al., 2008). We neither observed any improvement nor exacerbation of liver function produced by, or associated with, celecoxib administration in the CCl_4-treated rats in our study. In our own view, the reason for these diverse and conflicting observations may not be unconnected with the complex roles of the COX-2-prostanoid pathway in the pathogenesis and pathophysiology of various liver diseases (Hu, 2003). Li et al. (2009) have therefore suggested that given the importance of COX-2-derived PGs in several key aspects of liver pathobiology, including hepatocyte survival, liver regeneration, chronic hepatitis, liver injury, and hepatocarcinogenesis, studies are required to detail the mechanisms of COX-2 and PG actions in different liver diseases and animal models.

Interestingly, however, we observed that celecoxib significantly attenuated hypercholesterolaemia and LPO associated with the liver injury in this study. Hypercholesterolemia, a prominent metabolic disorder, is well recognized as a major risk factor for coronary heart disease and atherosclerosis. Although elevated serum lipid levels are often observed in liver injury (Senthilkumar et al., 2003; Aiyar et al., 1964), attention, however, has rarely been paid to its precise contribution to the pathogenesis or progression of hepatic steatosis, inflammation, and fibrosis. A few experimental studies have demonstrated an association between the intake of high cholesterol and high-fat diets with liver steatosis and inflammation (Buyssens et al., 1996; Tous et al., 2005). In addition to an epidemiological study suggesting hypercholesterolemia as an independent risk factor for liver disease, this metabolic disorder has also been reported to increase susceptibility to virus-induced immunopathological liver disease (Ludewing et al., 2001; Pendino et al., 2005). Recently, Ferre et al. (2009), demonstrated that hypercholesterolemic apolipoprotein E–deficient (Apo $E^{-/-}$) mice were more susceptible to developing severe liver injury, suggesting that increased cholesterol products also play a contributory role in accelerating the progression of inflammatory and fibrogenic responses in the liver, in addition to vascular disease. They also provided evidence that oxidized cholesterol products (i.e., oxysterols) representative of those detected in the liver from hypercholesterolemic Apo $E^{-/-}$ mice trigger key molecular events involved in hepatic inflammation and fibrogenesis in liver cells that were consistent with those found *in vivo* in hypercholesterolemic Apo $E^{-/-}$ mice.

The observed significant reduction in hypercholesterolemia associated with CCl_4-mediated toxic injury to the liver in the celecoxib-treated rats in this study appears to be in line with previous studies that have shown that increased expression of COX-2 contributes

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to atherosclerosis (van der Wal et al., 1994; Praticó and FitzGerald, 1996; Fukunaga et al., 1993). Our result also seems to corroborate observations from a recent study where celecoxib exerted a beneficial lowering effect on plasma levels of inflammatory markers, risky lipids, and on the size of atheromatous patches, both in an experimental model of hyperlipidemia and patients with unstable angina. The selective inhibition of COX-2 with celecoxib also attenuated cardiovascular events in the patients with unstable angina, and this was attributed to the reduction in the atheromatous patches as well as anti-inflammatory and hypolipidemic effects (Abdel-Fadeil et al., 2011).

Although celecoxib significantly lowered plasma cholesterol in this study, the severe centrilobular hepatic necrosis, inflammation (characterized by mononuclear cell infiltration), and steatosis were hardly controlled, especially at twice the therapeutic dose. Although the reason for this is presently not clear, this may not be unconnected with exacerbated liver injury reported to be more evident in hypercholesterolemic mice when challenged with a potent hepatotoxic agent, such as CCl_4 (Gonzalez-Peiz et al., 2006; Planaguma et al., 2005). Further, the decrease in hepatic LPO we observed, on the other hand, appears to reveal a slowing of the oxidative modification of the accumulated lipids in the liver, and suggests that celecoxib may provide some long-term benefit in this condition. In line with our present observation, COX-2 inhibition with rofecoxib has also been linked with reduction in LPO in rats with sepsis-induced liver damage (Kara et al., 2004). The COX-2, a proinflammatory factor, is known to play an important role in tissue damage during the early inflammatory response, and it is increasingly recognized that oxidative stress can elicit inflammatory responses in tissues (Deng et al., 2006; Li and Matsumura, 2008). The significant inhibition of LPO produced by celecoxib in this study further suggest that oxidative stress may be an important trigger of COX-2 expression and the eventual inflammatory responses that contribute to tissue damage during CCl_4 toxicity. Similarly, because oxidative modification of lipids, such as low-density lipoprotein cholesterol, is a major risk factor in the development of atherosclerosis (Prasad and Lee, 2001; Alkhouri et al., 2010), our present findings corroborate reports from other studies that associated treatment with selective COX-2 inhibitors with the prevention of the development of atherosclerotic lesions (Jacob et al., 2008; Abdel-Fadeil et al., 2011).

Conclusion

We therefore conclude that though the exact role of COX-2 inhibition and its therapeutic efficacy remain doubtful in liver disease, celecoxib and possibly other inhibitors of COX-2 may provide a useful option in controlling hypercholesterolemia and tissue damage associated with LPO during this condition.

Acknowledgments

The authors acknowledge the support of Prof. E.O. Ogunyemi, the Head of the Department of Chemical Pathology and Immunology of the Olabisi Onabanjo University Teaching Hospital, at the time of this study. The assistance of Ms. N.T. Oresanya of the Department of Pharmacology is also gratefully acknowledged.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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