JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. 2006; **26**: 178–186
Published online 8 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jat.1122

Preventive effect of neutropenia on carbon tetrachloride-induced hepatotoxicity in rats

Yoshiji Ohta,[1,*] Yoichiro Imai,[2] Tatsuya Matsura,[3] Akira Kitagawa[4] and Kazuo Yamada[3]

[1] Department of Chemistry, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan
[2] Department of Clinical Biochemistry, Fujita Health University College, Toyoake, Aichi 470-1192, Japan
[3] Department of Pathophysiology and Therapeutic Science, Faculty of Medicine, Tottori University, Yonagao, Tottori 683-8503, Japan
[4] Department of Nutrition, Faculty of Wellness, Chukyo Women's University, Ohbu, Aichi 474-8651, Japan

Received 27 July 2005; Accepted 27 September 2005

ABSTRACT: The preventive effect of neutropenia on carbon tetrachloride (CCl_4)-induced hepatotoxicity was examined in rats. In rats treated once with CCl_4 (1 ml kg^{-1}, i.p.), the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indices of liver cell damage, and the hepatic activity of myeloperoxidase (MPO), an index of tissue neutrophil infiltration, increased at 6 h after the intoxication and further increased at 24 h. The liver of CCl_4-treated rats showed an increase in the concentration of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, and decreases in superoxide dismutase (SOD) activity and reduced glutathione (GSH) concentration at 6 h after the intoxication followed by a further increase in TBARS concentration and further decreases in SOD activity and GSH concentration at 24 h with increased xanthine oxidase (XO) activity at 24 h. Neutropenic treatment with anti-rat neutrophil antiserum (2 ml kg^{-1}, i.p.) at 0.5 h after CCl_4 intoxication attenuated the increases in serum ALT and AST activities and hepatic MPO activity and TBARS concentration and the decreases in hepatic SOD activity and GSH concentration found at 6 and 24 h after CCl_4 intoxication and the increase in hepatic XO activity found at 24 h after the intoxication. This neutropenia reduced the necrotic and degenerative changes with inflammatory cell infiltration in the liver cell of CCl_4-treated rats. These results indicate that neutropenia prevents CCl_4-induced hepatotoxicity in rats by attenuating the disruption of hepatic reactive oxygen species metabolism mediated by neutrophils accumulating in the liver tissue. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: carbon tetrachloride; hepatotoxicity (rat); neutropenia; myeloperoxidase; reduced glutathione; superoxide dismutase; xanthine oxidase; reactive oxygen species metabolism

Introduction

It is well known that carbon tetrachloride (CCl_4) induces hepatotoxicity in humans and experimental animals. According to the present view of the initial developmental process of CCl_4-induced hepatotoxicity, the processes are dominated by factors such as CCl_4 activation to trichloromethyl radical ($CCl_3^{\bullet}$) and trichloromethyl peroxy radical, the covalent binding of $CCl_3^{\bullet}$ to membrane lipids and proteins, and the hydrogen abstraction from polyunsaturated fatty acids by trichloromethyl peroxy radical and $CCl_3^{\bullet}$ to initiate lipid peroxidation (Recknagel *et al*., 1989; Weber *et al*., 2003).

Lipid peroxidation in cell membranes occurs via reactive oxygen species (ROS), such as superoxide radical (O_2^{-}) and hydroxyl radical ($OH^{\bullet}$), and lipid peroxidation and ROS cause destruction of cell components and cell death (Gutteridge and Halliwell, 1990). Several reports have shown that hepatic lipid peroxidation increases with the development of acute liver injury in rats treated once with CCl_4 (Miyazawa *et al*., 1990; Ohta *et al*., 1995, 1997, 2000, 2003; Hartley *et al*., 1999; Muriel *et al*., 2001; Sun *et al*., 2001; Campo *et al*., 2004). There are many reports showing that a single treatment of rats with CCl_4 causes disruption of the hepatic antioxidant defense systems associated with antioxidants such as reduced glutathione (GSH) and ascorbic acid, which scavenge ROS, and antioxidant enzymes such as superoxide dismutase (SOD), an enzyme to dismute O_2^{-} to hydrogen peroxide (H_2O_2) and O_2, catalase, an enzyme that decomposes H_2O_2 to H_2O and O_2, and glutathione reductase, an enzyme that converts oxidized glutathione to GSH using NADPH as a coenzyme, during the development of liver injury (Ohta *et al*., 1995, 1997, 2000, 2003, 2004; Muriel *et al*., 2001; Sun *et al*., 2001; Campo *et al*., 2004). In addition, our recent report has shown that ROS derived from xanthine oxidase (XO) contributes to the progression of acute liver injury in rats treated with CCl_4 (Ohta *et al*., 2003). Inflammatory processes in which Kupffer cells, i.e. the resident macrophages of the liver, cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10, and adhesion molecules such as intercellular adhesion molecule-1 are implicated in the development of CCl_4-induced acute

* Correspondence to: Dr Y. Ohta, Department of Chemistry, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan.
E-mail: yohta@fujita-hu.ac.jp

hepatic injury (Czaja *et al*., 1989, 1995; Armendariz-Borunda *et al*., 1991; Edwards *et al*., 1993; Tower *et al*., 1994; DeCicco *et al*., 1998; Louis *et al*., 1998; Neubauer *et al*., 1998; Muriel *et al*., 2001; Simeonova *et al*., 2001; Hojo *et al*., 2002; Nakamoto *et al*., 2003). It is known that neutrophils, i.e. polymorphonuclear leukocytes, are accumulated in the damaged liver of rats and mice treated once with CCl_4 (Louis *et al*., 1998; Campo *et al*., 2004). Badger *et al*. (1996) have shown that neutropenia produced by treatment with anti-neutrophil antiserum reduces the severity of CCl_4-induced liver injury in rats pretreated with all-*trans*-retinol to potentiate the liver injury via Kupffer cells. Edwards *et al*. (1993) supported the hypothesis that Kupffer cells participate in the mechanism of CCl_4-induced liver injury possibly by the release of chemoattractants for neutrophils, and have shown that CCl_4-induced liver injury is diminished when rats are made neutropenic. However, it is still not known how neutropenia prevents CCl_4-induced hepatotoxicity in rats. It has been shown that O_2^- generated by NADPH oxidase in activated neutrophils is involved in liver injury development after hemorrhage/resuscitation in mice and in the development of endotoxin-induced liver injury in mice (Lehnert *et al*., 2003; Gujral *et al*., 2004). It has been reported that activated neutrophils injure the isolated, perfused rat liver by an ROS-dependent mechanism (Dahm *et al*., 1991). Myeloperoxidase (MPO) is mainly present in neutrophils among the inflammatory cells and the enzyme produces hypochlorous acid (HOCl), a powerful oxidant, in the presence of H_2O_2 and Cl^- (Kettle and Winterbourn, 1997). Bilzer and Lauterburg (1991) reported that HOCl and chloramines, which are generated by the reaction of HOCl and amines such as ammonia and taurine, damage the isolated, perfused rat liver.

Therefore, the purpose of the present study was to clarify how neutropenia prevents CCl_4-induced hepatotoxicity in rats. The effect of neutropenia produced by treatment with anti-neutrophil antiserum on the development of hepatic injury in rats with a single CCl_4 intoxication was examined and also the changes in the hepatic activities of MPO, an index of hepatic neutrophil infiltration (Duval *et al*., 1990; Schierwagen *et al*., 1990; Komatsu *et al*., 1992), XO, and SOD activities and the hepatic concentrations of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, and GSH. In addition, the effect of neutropenia on the development of CCl_4-induced acute liver injury was checked by histological examination.

Materials and Methods

Chemicals

Leupeptin, 2,2′,5,5′-tetramethylbenzidine (TMB), xanthine, bovine erythrocyte Cu,Zn-SOD and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, MO, USA); dithiothreitol, phenylmethylsulfonylfluoride, and milk XO and from Roche-Diagnostic Co. (Tokyo, Japan); CCl_4, ethylenediaminetetraacetic acid (EDTA), GSH, 2-thiobarbituric acid, and other reagents, of the highest grade, were purchased from Wako Pure Chemical Ind., Ltd (Osaka, Japan). All chemicals were used without further purification.

Animals

Male Wistar rats aged 5 weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan). The animals were maintained under a daily controlled 12 h light, 12 h dark lighting cycle at 23 °C and 50% humidity with free access to rat chow (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water. All animals received humane care in compliance with the Guidelines for the Management of Laboratory Animals in Fujita Health University.

Induction of Hepatic Injury

The CCl_4 was diluted twice with olive oil. Fed rats (6 weeks old) received a single intraperitoneal (i.p.) injection of CCl_4 at a dose of 1 ml kg^{-1} body weight (BW), that is, 2 ml kg^{-1} BW of the 50% CCl_4 solution, to produce liver injury. Rats without CCl_4 injection received an equal volume of olive oil in the same manner. These animals were starved with free access to water after CCl_4 injection, as described in our previous reports (Ohta *et al*., 2000, 2003, 2004).

Treatment with Anti-neutrophil Antiserum

Rats with and without CCl_4 intoxication received a single i.p. injection of anti-rat neutrophil antiserum (Funakoshi Co, Tokyo, Japan) at a dose of 2 ml kg^{-1} BW at 0.5 h after the intoxication. Rats without anti-rat neutrophil antiserum injection received the same volume of rabbit serum (Funakoshi Co, Tokyo, Japan) at the same time point.

Sample Preparation

Rats were killed after collecting blood from the inferior vena cava under ether anesthesia at 0.5, 6 and 24 h after CCl_4 injection. Immediately after killing, the livers were perfused with ice-cold 0.9% NaCl through the portal vein to remove as much residual blood as possible from the tissue and then removed from the body. The isolated liver was washed well in ice-cold 0.9% NaCl, blotted on a filter, weighed and frozen on dry ice as soon as possible.

The collected blood was kept on ice for 30 min and then separated into serum by centrifugation at 4 °C. The isolated liver and serum were kept at −80 °C until use. For liver XO assays, a part of the right large lobe of each liver was homogenized in 9 volumes of an ice-cold buffered solution (pH 7.8) containing 0.05 M potassium phosphate, 0.1 M EDTA, 0.5 mM dithiothreitol, 0.5 mg ml^{-1} leupeptin and 0.2 mM phenylmethylsulfonylfluoride using a glass homogenizer with a Telfon pestle as described previously (Ohta *et al*., 2003, 2004). The homogenate was dialysed against 100 volumes of the same buffered solution at 4 °C for 60 min using a microdialysis device (MWCO = 3500 Da) (Bio-Tec International Inc., Bellevue, WA, USA). For determination of hepatic LPO and GSH and MPO and SOD activities, another part of the right large lobe of each liver was homogenized in 9 volumes of ice-cold 0.15 M KCl containing 1.0 mM EDTA using a glass homogenizer with a Telfon pestle. For MPO and SOD assays, the prepared homogenate was sonicated on ice twice for 30 s using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo, Japan). The sonicated homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C. The resultant supernatant was dialysed against 100 volumes of 0.05 M Tris-HCl buffer (pH 7.4) for 60 min using the above-described microdialysis device.

Assays of Serum Transaminases and Hepatic Components and Enzymes

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using a commercial kit, Iatorzyme TA-L_Q (Dai-Iatron Co., Tokyo, Japan). Both activities were expressed as international units (IU l^{-1}). Hepatic TBARS was determined by the method of Ohkawa *et al*. (1979) using the thiobarbituric acid reaction except that 1.0 mM EDTA was added to the reaction mixture. The concentration of TBARS is expressed as the amount of malondialdehyde (MDA) equivalents. Hepatic GSH was determined by the method of Sedlak and Lindsay (1968) using Ellman's reagent and GSH as a standard. Hepatic MPO was assayed as follows: the dialysed supernatant was incubated at 60 °C for 2 h to increase the recovery of MPO in the liver tissues according to the method of Schierwagen *et al*. (1990). This heat treatment caused a complete inactivation of catalase, which is known to influence the assay of MPO in liver tissues. MPO activity in the heat-treated supernatant was assessed by measuring H_2O_2-dependent oxidation of TMB at 37 °C according to the method of Suzuki *et al*. (1983). This TMB oxidation was measured spectrophotometrically at 655 nm. One unit of this enzyme activity was expressed as the amount of enzyme causing a change in absorbance of 1.0 per min at 650 nm. Hepatic SOD was assayed by the method of Oyangui (1984). SOD activity was determined at 37 °C by the XO-NH_2OH method using purified bovine erythrocyte SOD (5000 units mg^{-1} solid) as a standard. This enzyme activity was expressed as the amount of the erythrocyte SOD showing activity equivalent to the determined activity. Hepatic XO was assayed at 30 °C by the method of Hashimoto (1974) using xanthine as a substrate. XO activity was assessed by measuring the increase in absorbance at 292 nm following the formation of uric acid. One unit (U) of XO activity is defined as the amount of enzyme forming 1 μmol uric acid min^{-1}. Protein in liver tissue samples was measured by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Histological Examination

Liver samples were taken from the central part of the right large lobe of CCl_4-treated and untreated rats with and without anti-neutrophil antiserum treatment at 24 h after the intoxication. The samples were fixed with 10% formalin in phosphate buffered saline for 24 h and then washed with tap water, dehydrated in alcohols, and embedded in paraffin. Sections 6–7 μm thick were mounted in glass slides. Staining with hematoxylin and eosin (H-E) was performed on each slide and then histological examination was conducted under light microscopy.

Statistical Analysis

All values obtained are expressed as the mean ± standard deviation (SD). All data were statistically analysed by computerized statistical packages (StatView). Each mean value was compared by one-way analysis of variance and Fisher's protected significant difference for multi-comparison as the post hoc test. The level of significance was set at $P < 0.05$.

Results

Effect of Neutropenia on Serum ALT and AST Activities in CCl_4-treated Rats

In rats treated with CCl_4 (1 ml kg^{-1} BW), the serum ALT and AST activities were not changed at 0.5 h after the intoxication but both enzyme activities significantly increased at 6 h and further increased at 24 h (Fig. 1). When anti-neutrophil antiserum (2 ml kg^{-1} BW) was administered to CCl_4-intoxicated rats at 0.5 h after the intoxication, the increases in serum ALT and AST activities found at 6 and 24 h after the intoxication were significantly depressed; the ALT and AST activities in the CCl_4-treated group with neutropenic treatment were 3.9- and 6.1-fold, respectively, higher than those in the

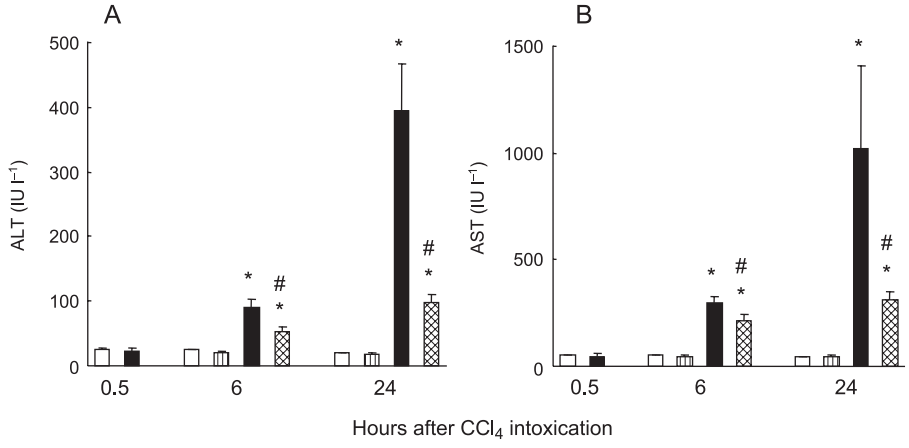

Figure 1. Effect of neutropenic treatment on serum ALT (A) and AST (B) activities in CCl_4-treated and untreated rats. Rats received a single i.p. injection of either CCl_4 (1 ml kg^{-1} BW) or olive oil used as a vehicle. At 0.5 h after CCl_4 intoxication, they received a single i.p. injection of either anti-neutrophil antiserum (2 ml kg^{-1} BW) or rabbit serum (2 ml kg^{-1} BW). The animals were killed 0.5, 6 and 24 h after CCl_4 intoxication. Open bar, untreated control rats; closed bar, rats treated with CCl_4 alone; striped bar, CCl_4-untreated rats with neutropenia; crosshatched bar, CCl_4-treated rats with neutropenia. Each value is the mean ± SD (n = 5–10). * $P < 0.05$ (vs control rat); # $P < 0.05$ (vs corresponding rats treated with CCl_4 alone)

control group at 24 h after the intoxication (Fig. 1). The same neutropenic treatment had no effect on serum ALT and AST activities in CCl_4-untreated rats at the time points corresponding to 6 and 24 h after CCl_4 intoxication (Fig. 1).

Effect of Neutropenia on Hepatic Histological Changes in CCl_4-treated Rats

The liver sections stained by H-E in CCl_4-treated rats with and without neutropenic treatment and untreated control rats were examined for necrosis and extent of inflammation at 24 h after the intoxication. Hepatocytes in the untreated control group showed few histological changes (Fig. 2A). Hepatocytes in the centrilobular area of the CCl_4-treated group presented necrotic and degenerative changes with severe inflammatory cell infiltration (Fig. 2B). In contrast, hepatocytes in the centrilobular area of the CCl_4-treated group with neutropenic treatment presented many fewer necrotic and degenerative changes and little inflammatory cell infiltration (Fig. 2C).

Effect of Neutropenia on Hepatic MPO Activity in CCl_4-treated Rats

The hepatic MPO activity in CCl_4-treated rats was not changed at 0.5 h after the intoxication but the enzyme activity increased at 6 h and further increased at 24 h; the intoxicated group had 2.4- and 5.6-fold higher hepatic MPO activity than the control group at 6 and 24 h after the intoxication, respectively (Fig. 3). There was no significant difference in the hepatic MPO activity between the CCl_4-treated group with neutropenic treatment and the control group at 6 h after the intoxication and the CCl_4-treated group with neutropenic treatment had a 3.2-fold higher hepatic MPO activity than the control group at 24 h after the intoxication (Fig. 3). In CCl_4-untreated rats with the same neutropenic treatment, the hepatic MPO activity significantly decreased at the time points corresponding to 6 and 24 h after CCl_4 intoxication (Fig. 3).

Effect of Neutropenia on Hepatic TBARS and GSH Concentrations and XO and SOD Activities in CCl_4-treated Rats

In CCl_4-treated rats, a significant increase in hepatic TBARS concentration and a significant decrease in hepatic GSH concentration occurred at 6 and 24 h, but not at 0.5 h, after the intoxication (Fig. 4A, B). The hepatic TBARS concentrations in the CCl_4-treated group were 1.8- and 4.7-fold higher than that in the control group at 6 and 24 h after the intoxication, respectively (Fig. 4A). The hepatic GSH concentrations in the intoxicated group were 57.3% and 44.7% of that in the control group at 6 and 24 h after the intoxication, respectively (Fig. 4B). Neutropenic treatment significantly attenuated the increase in hepatic TBARS concentration and the decrease in hepatic GSH concentration found at 6 and 24 h after CCl_4 intoxication (Fig. 4A, B). The CCl_4-treated group with the same neutropenic treatment had 1.4- and 2.1-fold higher hepatic TBARS concentration than the control group at the time points corresponding to 6 and 24 h after the intoxication, respectively (Fig. 4A).

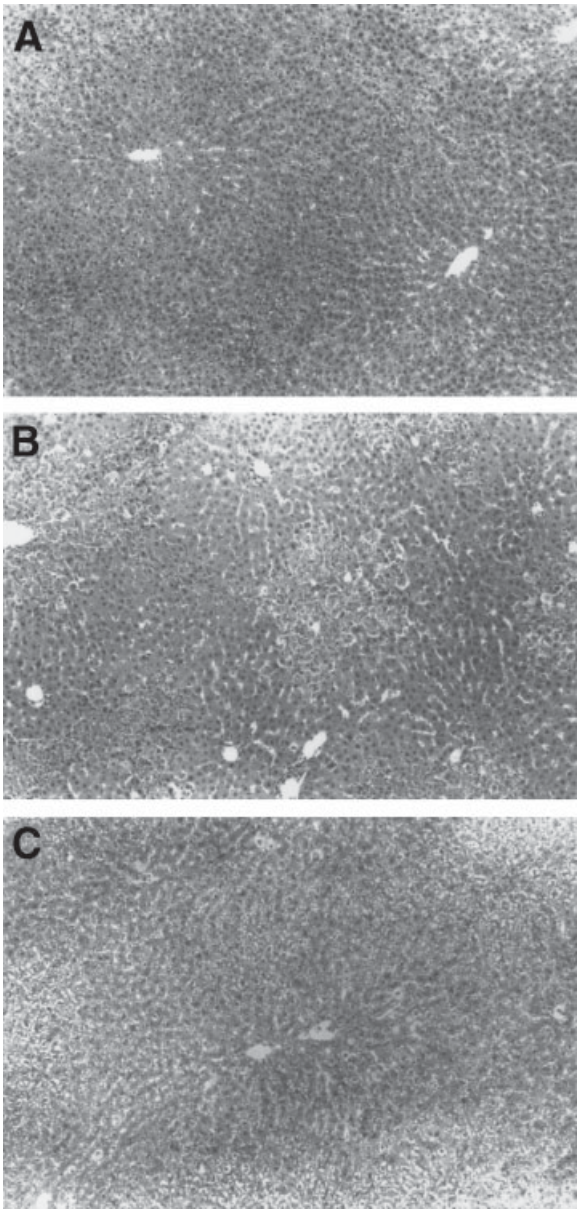

Figure 2. Histology of the rat liver cell at 24 h after CCl_4 intoxication. (A) untreated control group: little histological change was observed; (B) CCl_4-treated group: necrotic and degenerative changes with severe inflammatory cell infiltration were observed; (C) CCl_4-treated group with neutropenic treatment which was conducted 0.5 h after the intoxication: dramatic decreases in necrotic and degenerative changes and inflammatory cell infiltration were seen. (H-E, magnification × 50)

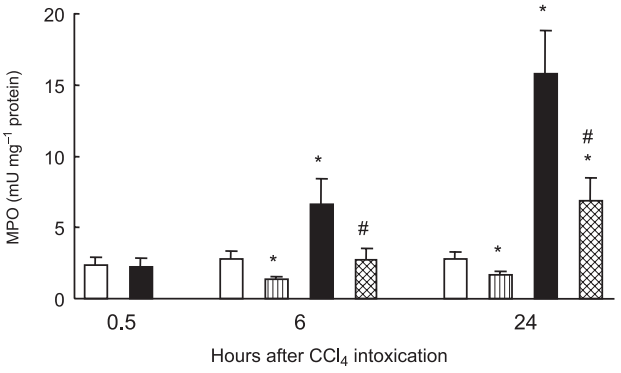

Figure 3. Effect of neutropenic treatment on hepatic MPO activity in CCl_4-treated and untreated rats. Rats received a single i.p. injection of either CCl_4 (1 ml kg^{-1} BW) or olive oil used as a vehicle. At 0.5 h after CCl_4 intoxication, they received a single i.p. injection of either anti-neutrophil antiserum (2 ml kg^{-1} BW) or rabbit serum (2 ml kg^{-1} BW). The animals were killed 0.5, 6 and 24 h after CCl_4 intoxication. Open bar, untreated control rats; closed bar, rats treated with CCl_4 alone; striped bar, CCl_4-untreated rats with neutropenia; crosshatched bar, CCl_4-treated rats with neutropenia. Each value is the mean ± SD (n = 5–10). * $P < 0.05$ (vs control rat); # $P < 0.05$ (vs corresponding rats treated with CCl_4 alone)

The CCl_4-treated group with neutropenic treatment had 85.4% and 77.6% of hepatic GSH concentration in the control group at 6 and 24 h after the intoxication, respectively (Fig. 4B). In CCl_4-untreated rats with the same neutropenic treatment, the hepatic TBARS concentration significantly decreased at the time points corresponding to 6 and 24 h after CCl_4 intoxication, but the hepatic GSH concentration did not change at both time points (Fig. 4A, B). Hepatic XO activity in the CCl_4-treated rats significantly increased at 24 h, but not at 0.5 and 6 h, after the intoxication, while hepatic SOD activity in the CCl_4-treated rats significantly decreased at 6 and 24 h, but not at 0.5 h, after the intoxication (Fig. 4C, D). The hepatic XO activity in the CCl_4-treated group was 5.2-fold higher than that in the control group at 24 h after the intoxication (Fig. 4C). The CCl_4-treated group had 80.5% and 54.8% of hepatic SOD activity in the control group at 6 and 24 h after the intoxication, respectively (Fig. 4D). Neutropenic treatment significantly attenuated the increase in hepatic XO activity found at 24 h after CCl_4 intoxication and the decrease in hepatic SOD activity found at 6 and 24 h after the intoxication (Fig. 4C, D). The CCl_4-intoxicated group with neutropenic treatment had a 3.2-fold higher hepatic XO activity than the control group at 24 h after the intoxication (Fig. 4C). The hepatic SOD activity in the CCl_4-treated group with neutropenic treatment did not differ from that in the control group at 6 h after the intoxication and the CCl_4-treated group with neutropenic treatment had a 82.2% of hepatic SOD activity in the control group at 24 h after the intoxication (Fig. 4D). The same neutropenic treatment did not affect hepatic XO and SOD activities in the CCl_4-untreated rats at the time points corresponding to 6 and 24 h after CCl_4 intoxication (Fig. 4C, D).

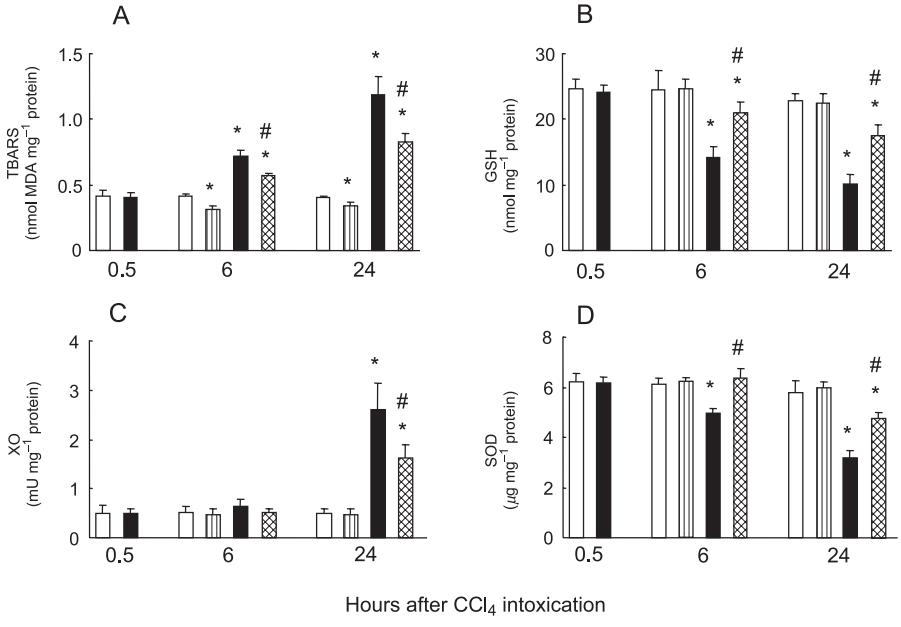

Figure 4. Effect of neutropenic treatment on hepatic TBARS (A) and GSH (B) concentrations and XO (C) and SOD (D) activities in CCl_4-treated and untreated rats. Rats receive a single i.p. injection of either CCl_4 (1 ml kg^{-1} BW) or olive oil used as a vehicle. At 0.5 h after CCl_4 intoxication, they received a single i.p. injection of either anti-neutrophil antiserum (2 ml kg^{-1} BW) or rabbit serum (2 ml kg^{-1} BW). The animals were killed 0.5, 6 and 24 h after CCl_4 intoxication. Open bar, untreated control rats; closed bar, rats treated with CCl_4 alone; striped bar, CCl_4-untreated rats with neutropenia; crosshatched bar, CCl_4-treated rats with neutropenia. Each value is a mean ± SD (n = 5–10). * $P < 0.05$ (vs control rat); # $P < 0.05$ (vs corresponding rats treated with CCl_4 alone)

Discussion

In the present study, acute hepatic injury in rats treated with CCl_4 (1 m kg^{-1} BW), judged by the serum levels of ALT and AST, indices of liver cell damage, developed with an increase in the hepatic activity of MPO, an index of hepatic neutrophil infiltration (Duval *et al*., 1990; Schierwagen *et al*., 1990; Komatsu *et al*., 1992). When CCl_4-treated rats received anti-neutrophil antiserum treatment before the occurrence of hepatic injury, hepatic injury development was prevented with attenuation of the increased hepatic MPO activity. This preventive effect of neutropenia on the development of CCl_4-indced hepatic injury was confirmed by histological observation. Thus, the present study has clearly shown that neutropenia prevents CCl_4-induced hepatotoxicity in rats.

It has been shown that both enhanced lipid peroxidation and GSH depletion occur with the development of injury in the liver of rats treated once with CCl_4 (Ohta *et al*., 1995, 1997, 2000, 2003). Activated neutrophils produce ROS via NADPH oxidase (Takahashi *et al*., 1991). It is known that O_2^- generated by activated neutrophils enables these cells to enhance their production of HOCl (Kettle and Winterbourn, 1990). HOCl is produced by MPO released from activated neutrophils in the presence of H_2O_2 and Cl^- (Kettle and Winterbourn, 1997). It has been shown that neutrophils mediate lipid peroxidation through the production of O_2^- via NADPH oxidase in the cells (Casani *et al*., 1997; Zimmerman *et al*., 1997). It has also been shown that MPO mediates lipid peroxidation in the presence of H_2O_2 and halide ions (Stelmaszynska *et al*., 1992; Zhang *et al*., 2002). GSH is known to react with O_2^- and HOCl *in vitro* (Ross *et al*., 1985; Prütz, 1996; Puller *et al*., 2001). GSH is also known to be consumed in the MPO-H_2O_2-Cl^- system (Winterbourn, 1985). In the present study, the hepatic concentration of TBARS, an index of lipid peroxidation, in rats treated with CCl_4 increased at an early stage of hepatic injury and further increased at a progressed stage of the injury, as reported previously (Ohta *et al*., 2000, 2003). The increase in hepatic TBARS concentration with the development of CCl_4-induced hepatic injury was attenuated by neutropenic treatment. In addition, CCl_4-untreated rats with neutropenic treatment showed a decrease in the hepatic TBARS concentration. In CCl_4-treated and untreated rats with neutropenic treatment, the decrease in hepatic TBARS concentration was consistent with the decrease in hepatic MPO activity. Hepatic GSH concentration in rats with CCl_4 intoxication decreased at an early stage of hepatic injury and further decreased at a progressed stage of the injury, as reported previously (Ohta *et al*., 2000, 2003). The decrease in hepatic GSH concentration with the development of CCl_4-induced hepatic injury was attenuated by neutropenic treatment.

However, neutropenic treatment had no effect on the hepatic GSH concentration in CCl_4-untreated rats. These findings suggest that neutropenia prevents CCl_4-induced hepatotoxicity in rats by attenuating lipid peroxidation and GSH depletion mediated by neutrophils accumulating in the liver tissue.

It has been shown that XO-derived ROS contributes to the progression of CCl_4-induced acute liver injury in rats (Ohta *et al*., 2003). In the present study, rats intoxicated with CCl_4 had increased hepatic XO activity at a progressed stage of hepatic injury, as shown in our previous reports (Ohta *et al*., 2003, 2004). Neutropenic treatment attenuated the increase in hepatic XO activity found in CCl_4-treated rats but it did not affect the hepatic XO activity in CCl_4-untreated rats. These results suggest that neutropenia prevents the development of CCl_4-induced acute hepatic injury in rats by reducing the neutrophil-mediated increase in hepatic XO activity. It has been suggested that the CCl_4-induced increase in hepatic XO activity is due to the conversion of xanthine dehydrogenase (XD) to XO in the ischemic or hypoxic liver (Ohta *et al*., 2003). The conversion of XD to XO in liver tissues is known to be caused by a proteolytic mechanism and/or the oxidation of sulfhydryl groups present in the protein (Corte and Stirpe, 1972; Nishino and Tamura, 1991). As described above, neutropenic treatment attenuated the decrease in hepatic GSH concentration found in the CCl_4-treated rats. Activated neutrophils not only produce ROS but also release proteases from the cells (Travis, 1988; Takahashi *et al*., 1991). Therefore, it is suggested that neutrophils accumulating in the liver tissue of rats treated with CCl_4 convert XD to XO not only via the oxidation of sulfhydryl groups present in the protein of XD by ROS generated by the cells, but also via proteases released from the cells in the liver tissue, resulting in an increase in XO activity in the liver of CCl_4-treated rats.

In the present study, hepatic SOD activity in rats with CCl_4 intoxication decreased at an early stage of acute hepatic injury and further decreased at a progressed stage of the injury, as reported previously (Ohta *et al*., 1995, 1997, 2003, 2004). Neutropenic treatment attenuated the decrease in hepatic SOD activity with the development of CCl_4-induced hepatic injury. However, neutropenic treatment had no effect on hepatic SOD activity in CCl_4-untreated rats. Previous reports have shown that in the liver of rats treated once with CCl_4 the activity of Cu,Zn-SOD present in the cytosol, but not the activity of Mn-SOD present in the mitochondria, decreases with hepatic injury development (Ohta *et al*., 2003, 2004). Cu,Zn-SOD is known to be inactivated by HOCl and by the MPO-H_2O_2-Cl^- system (Aruoma and Halliwell, 1987; Sharonov and Churilova, 1992; Auchère and Capeillère-Blandin, 2002). Escobar *et al*. (1996) have shown that alkyl-peroxyl radicals generated by 2,2′-azobis(2-amidinopropane), which is used as a source of radicals for lipid peroxidation, inactivate Cu,Zn-SOD *in vitro*. As described above, activated neutrophils and MPO mediate lipid peroxidation *in vitro* (Stelmaszynska *et al*., 1992; Casani *et al*., 1997; Zimmerman *et al*., 1997; Zhang *et al*., 2002). These findings suggest that neutropenia prevents CCl_4-induced hepatotoxicity in rats by attenuating the neutrophil-mediated reduction of hepatic SOD activity.

There are some reports showing that proteases released from activated neutrophils contribute to ischemia/reperfusion-induced liver injury in rats (Kushimoto *et al*., 1996; Yamaguchi *et al*., 1997; Chen *et al*., 1999). It has been shown that ischemia/reperfusion-like blood flow occurs during the development of injury in the liver of rats treated once with CCl_4 (Makino, 1990). Therefore, the possibility cannot be ruled out that neutropenia prevents hepatotoxicity in rats treated with CCl_4 by attenuating the release of proteases from neutrophils accumulating in the liver tissue.

When Kupffer cells respond to hepatocellular stress, the cells not only produce ROS but also release pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, which recruit neutrophils into liver cells through expression of adhesion molecules (Vrba and Modriansk$\acute{y}$, 2002). It has been implicated that cytokines such as TNF-α, IL-1, LI-6 and interleukin-10 contribute to liver damage in rats with a single CCl_4 treatment (Czaja *et al*., 1989, 1995; DeCicco *et al*., 1998; Louis *et al*., 1998; Neubauer *et al*., 1998; Simeonova *et al*., 2001; Hojo *et al*., 2002; Nakamoto *et al*., 2003; Campo *et al*., 2004). Edwards *et al*. (1993) have indicated that Kupffer cells cause liver damage in rats with a single CCl_4 treatment possibly by the release of chemoattractants for neutrophils. It is known that inhibition of Kupffer cells attenuates liver damage with prevention of increased hepatic lipid peroxidation in rats treated once with CCl_4 (Muriel *et al*., 2001). Taken together with these findings and the results of the present study, it seems likely that neutropenia prevents CCl_4-induced hepatotoxicity in rats by reducing hepatic lipid peroxidation and damage mediated by Kupffer cells in an indirect manner.

In conclusion, the results of the present study indicate that neutropenia produced by treatment with anti-neutrophil antiserum prevents CCl_4-induced hepatotoxicity in rats with attenuation of the neutrophil-mediated disruption of hepatic ROS metabolism associated with increased hepatic lipid peroxidation and MPO and XO activities and decreased hepatic GSH concentration and SOD activity.

References

Armendariz-Borunda J, Seyer JM, Postlethwaite AE, Kang AH. 1991. Kupffer cells from carbon tetrachloride-injured rat livers produce chemotactic factors for fibroblasts and monocytes: The role of tumor necrosis factor-α. *Hepatology* **14**: 895–900.

Aruoma OI, Halliwell B. 1987. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochem. J.* **248**: 973–976.

Auchère F, Capeillère-Blandin C. 2002. Oxidation of Cu,Zn-superoxide dismutase by the myeloperoxidase/hydrogen peroxide/chloride system: Functional and structural effects. *Free Radic. Res.* **36**: 1185–1198.

Badger DA, Sauer J-M, Hoglen NC, Jolley CS, Sipes IG. 1996. The role of inflammatory cells and cytochrome p450 in the potentiation of CCl_4-induced liver injury by a single dose of retinol. *Toxicol. Appl. Pharmaocol.* **141**: 507–519.

Bilzer M, Lauterburg BH. 1991. Effects of hypochlorous acid and chloramines on vascular resistance, cell integrity, and biliary glutathione disulfide in the perfused rat liver: modulation by glutathione. *J. Hepatol.* **13**: 84–89.

Campo GM, Avenoso A, Campo S, Ferlazzo AM, Micali C, Zanghí L, Calatroni A. 2004. Hyaluronic acid and chondroitin-4-sulphate treatment reduces damage in carbon tetrachloride-induced acute liver injury. *Life Sci.* **74**: 1289–1305.

Casani A, Ceni E, Salzano R, Biondi P, Parola M, Galli A, Foschi M, Caligiuri A, Pinzani M, Surrenti C. 1997. Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: Role of nitric oxide. *Hepatology* **25**: 361–367.

Chen H-M, Chen J-C, Shyr M-H, Chen M-F, Hwang T-L, Fan L-L, Chi T-Y, Chi CP. 1999. Neutrophil elastase inhibitor (ONO-5046) attenuates reperfusion-induced hepatic microcirculatory derangement, energy depletion and lipid peroxidation in rats. *Shock* **12**: 462–467.

Corte ED, Stirpe F. 1972. The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type O) and purification of the enzyme. *Biochem. J.* **261**: 739–745.

Czaja MJ, Flanders KC, Biempica L, Klein C, Zern MA, Weiner FR. 1989. Expression of tumor necrosis factor-α and transforming growth factor-β1 in acute liver injury. *Growth Factors* **1**: 219–226.

Czaja MJ, Xu J, Alt E. 1995. Prevention of carbon tetrachloride-induced rat liver injury by soluble tumor necrosis factor receptor. *Gastroenterology* **108**: 1849–1854.

Dahm L, Schultze AE, Roth RA. 1991. Activated neutrophils injure the isolated, perfused rat liver by an oxygen radical-dependent mechanism. *Am. J. Pathol.* **139**: 1009–1020.

DeCicco LA, Rinkans LE, Tutor CG, Hornbrook R. 1998. Serum and liver concentrations of tumor necrosis factor α and interleukin-1β following administration of carbon tetrachloride to male rats. *Toxicol. Lett.* **98**: 115–121.

Duval DL, Howard D, McCalden TA, Billings RE. 1990. The determination of myeloperoxidase activity in liver. *Life Sci.* **47**: PL145–PL150.

Edwards MJ, Keller BJ, Kauffman FC, Thurman RG. 1993. The involvement of Kupffer cells in carbon tetrachloride toxicity. *Toxicol. Appl. Pharmacol.* **119**: 275–279.

Escobar JA, Rubio MA, Lissi EA. 1996. SOD and catalase inactivation by singlet oxygen and peroxyl radicals. *Free Radic. Biol. Med.* **20**: 285–290.

Gujral JS, Hinson JA, Farhood A, Jaeschke H. 2004. NADPH oxidase-derived oxidant is critical for neutrophil cytotoxicity during endotoxemia. *Am. J. Physiol.* **287**: G243–G252.

Gutteridge JMC, Halliwell B. 1990. The measurement and metabolism of lipid peroxidation in biological systems. *TIBS* **15**: 129–135.

Hartley DP, Kolaja KL, Reichard J, Peterson DR. 1999. 4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: Immunochemical detection and lobular localization. *Toxicol. Appl. Pharmacol.* **161**: 23–33.

Hashimoto S. 1974. A new spectrophotometric assay method of xanthine oxidase in crude tissue homogenate. *Anal. Biochem.* **62**: 426–433.

Hojo H, Kasakura T, Zuinen R, Aoki M, Yamaguchi M, Chikuma T, Sato M. 2002. Production of interleukin-6 and its implication of rats after subcutaneous injection of carbon tetrachloride. *J. Health Sci.* **48**: 134–139.

Kettle AJ, Winterbourn CC. 1990. Superoxide enhances hypochlorous acid production by stimulated neutrophils. *Biochim. Biophys. Acta* **1052**: 379–385.

Kettle AJ, Winterbourn CC. 1997. Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep.* **3**: 3–15.

Komatsu H, Koo A, Ghadishah E, Zeng H, Kuhlenkamp JF, Inoue M, Guth PH, Kaplowitz N. 1992. Neutrophil accumulation in ischemic reperfused rat liver: evidence for a role for superoxide free radical. *Am. J. Physiol.* **262**: G669–G676.

Kushimoto S, Okajima K, Uchida M, Murakami K, Harada N, Okabe H, Takatsuki K. 1996. Role of granulocyte elastase in ischemia/reperfusion injury of rat liver. *Crit. Care Med.* **24**: 1908–1912.

Lehnert M, Arteel GE, Smutney OM, Conzelmann LO, Zhong Z, Thurman RG, Lemasters JJ. 2003. Dependence of liver injury after hemorrhage/resuscitation in mice on NADPH oxidase-derived superoxide. *Shock* **19**: 345–351.

Louis H, van Laethem J-L, Wu W, Quertinmont E, Degraef, C, Van den Berg K, Demols A, Goldman M, Moine QL, Geerts A, Devière J. 1998. Interleukin-10 controls neutrophilic inflammation, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. *Hepatology* **28**: 1607–1615.

Lowry OH, Rosebrough NH, Farr AD, Randall RJ. 1951. Protein measurement with the Folin reagent. *J. Biol. Chem.* **193**: 265–273.

Makino T. 1990. Measurement of hepatic blood flow by the hydrogen gas clearance method. Experimental and clinical observations. *J. Nippon Med. School* (*Nippon Ikadaigaku Zasshi*) **57**: 137–146.

Miyazawa T, Suzuki T, Fujimoto K, Kaneda T. 1990. Phospholipid hydroperoxide accumulation in liver of rats intoxicated with carbon tetrachloride and its inhibition by dietary α-tocopherol. *J. Biochem.* **107**: 689–693.

Muriel P, Alba N, Pérez-Álvarez VM, Shibayama M, Tsutusmi VK. 2001. Kupffer cells inhibition prevents lipid peroxidation and damage induced by carbon tetrachloride. *Comp. Biochem. Physiol. Part C* **130**: 219–226.

Nakamoto N, Tada S, Kameyama K, Kitamura K, Kurita K, Saito Y, Saito H, Ishi H. 2003. A free radical scavenger, Edaravone, attenuates steatosis and cell death via reducing inflammatory cytokine production in rat acute liver injury. *Free Radic. Res.* **37**: 849–859.

Neubauer K, Eichhorst ST, Wilfling T, Buchenau M, Xia L, Ramadori G. 1998. Sinusoidal intercellular adhesion molecule-1 up-regulation precedes the accumulation of leukocyte function antigen-1-positive cells and tissue necrosis in a model of carbon tetrachloride-induced acute liver injury. *Lab. Invest.* **78**: 185–194.

Nishino T, Tamura I. 1991. The mechanism of conversion of xanthine dehydrogenase to oxidase and the role of the enzyme in reperfusion injury. *Adv. Exp. Med. Biol.* **309A**: 327–333.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues with thiobarbituric acid reaction. *Anal. Biochem.* **95**: 351–358.

Ohta Y, Kongo-Nishimura M, Imai Y, Kishikawa T. 2003. Contribution of xanthine oxidase-derived oxygen free radicals to the development of carbon tetrachloride-induced acute liver injury in rats. *J. Clin. Biochem. Nutr.* **33**: 89–93.

Ohta Y, Kongo-Nishimura M, Matsura T, Yamada K, Kitagawa A, Kishikawa T. 2004. Melatonin prevents disruption of hepatic reactive oxygen species metabolism in rats treated with carbon tetrachloride. *J. Pineal Res.* **36**: 10–17.

Ohta Y, Kongo M, Sasaki E, Nishida K, Ishiguro I. 2000. Therapeutic effect of melatonin on carbon tetrachloride-induced acute liver injury in rats. *J. Pineal Res.* **28**: 119–126.

Ohta Y, Nishida K, Sasaki E, Kongo M, Ishiguro I. 1997. Attenuation of disrupted hepatic active oxygen metabolism with the recovery of acute liver injury in rats intoxicated with carbon tetrachloride. *Res. Commun. Mol. Pathol. Pharmacol.* **95**: 191–207.

Ohta Y, Sasaki E, Nishida K, Hyashi T, Nagata M, Ishiguro I. 1995. Preventive effect of Dai-saiko-to (Da-Chai-Hu-Tang) extract on disrupted hepatic active oxygen metabolism in rats with carbon tetrachloride-induced liver injury. *Am. J. Chin. Med.* **23**: 53–64.

Oyanagui Y. 1984. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal. Bicohem.* **142**: 290–296.

Prütz W. 1996. Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substances. *Arch. Biochem. Biophys.* **332**: 110–120.

Puller JM, Vissers MCM, Winterbourn CC. 2001. Glutathione oxidation by hypochlorous acid in endothelial cells produces glutathione sulfonamide as a major product but not glutathione disulfide. *J. Biol. Chem.* **276**: 22120–22125.

Recknagel RO, Glende EA Jr, Dolak JA, Waller RL. 1989. Mechanisms of carbon tetrachloride toxicity. *Pharmacol. Ther.* **43**: 139–154.

Ross D, Cotgreave L, Modldes P. 1985. The interaction of reduced glutathione with active oxygen species generated by xanthine-oxidase-catalyzed metabolism of xanthine. *Biochim. Biophys. Acta* **841**: 278–282.

Schierwagen C, Bylund-Fellenius A-C, Lundberg C. 1990. Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J. Pharmacol. Methods* **23**: 179–186.

Sedlak J, Lindsay RH. 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **25**: 192–205.

Sharonov BP, Churilova IV. 1992. Inactivation and oxidative modification of Cu,Zn superoxide dismutase by stimulated neutrophils: The appearance of new catalytically active substances. *Biochem. Biophys. Res. Commun.* **189**: 1129–1135.

Simeonova PP, Gallucci RM, Hulderman T, Wilson R, Komminen C, Rao M, Luster MI. 2001. The role of tumor necrosis factor-α in liver toxicity, inflammation and fibrosis induced by carbon tetrachloride. *Toxicol. Appl. Pharmacol.* **177**: 112–120.

Stelmaszynska T, Kukovetz E, Egger G, Schaur RJ. 1992. Possible involvement of myeloperoxidase in lipid peroxidation. *Int. J. Biochem.* **24**: 121–128.

Sun F, Hamagawa E, Tsutsui C, Ono Y, Ogiri Y, Kojo S. 2001. Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver. *Biochim. Biophys. Acta* **1535**: 186–191.

Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. 1983. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.* **132**: 345–353.

Takahashi R, Edashige K, Sato EF, Inoue M, Matsuno T, Ustumi K. 1991. Luminol chemiluminescence and active oxygen generated by neutrophils. *Arch. Biochem. Biophys.* **285**: 325–330.

Tower RA, Reinke LA, Janzen EG, Yamashiro S. 1994. *In vivo* magnetic resonance imaging study of Kupffer cell involvement in CCl_4-induced hepatotoxicity in rats. *Can. J. Physiol. Pharmacol.* **72**: 441–446.

Travis J. 1988. Structure, function, and control of neutrophil proteinases. *Am. J. Med.* **84**: 37–43.

Vrba J, Modrianský M. 2002. Oxidation burst of Kupffer cells: Target for liver injury treatment. *Biomed. Papers* **146**: 15–20.

Weber LWD, Boll M, Stampfl A. 2003. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. *Crit. Rev. Toxicol.* **33**: 105–136.

Winterbourn CC. 1985. Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim. Biophys. Acta* **840**: 204–210.

Yamaguchi Y, Akizuki E, Ichiguchi O, Matsumura F, Goto M, Miyanari N, Mori K, Yamada S, Ogawa M. 1997. Neutrophil elastase inhibitor reduces neutrophil chemoattractant production after ischemia-reperfusion in rat liver. *Gastroenterology* **112**: 551–560.

Zhang R, Brennnan M-L, Shen Z, MacPherson JC, Schmitt D, Molenda CE, Hazen SL. 2002. Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. *J. Biol. Chem.* **277**: 46116–46122.

Zimmerman JJ, Ciesielski W, Lewandoski J. 1997. Neutrophil-mediated phospholipid peroxidation assessed by gas chromatography-mass spectroscopy. *Am. J. Physiol.* **273**: C653–C661.