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NF-κB Binding Activity and Cyclooxygenase-2 Expression in Persistent CCl_4-Treated Rat Liver Injury

The involvement of NF-κB binding activity is known to be important in the mechanism of acute liver injury and in the induction of cyclooxygenase (COX-2). This study was performed to evaluate NF-κB binding activity and the expression of COX-2 in chronic liver injury induced by carbon tetrachloride (CCl_4). Liver tissues from Sprague - Dawley rats were collected at 1, 3, 5, and 7th week after intraperitoneal injection of 0.1 mL of CCl_4/100 g body weight twice a week. Reactive oxygen species (ROS) were measured in the postmitochondrial fraction by dichlorofluorescein formation with a fluorescent probe. An electrophoretic mobility shift assay was performed for NF-κB binding activity. Western blot was performed to measure the level of COX-1, COX-2, p65, p50, and IκB proteins. ROS and NF-κB activity increased during the CCl_4-induced chronic liver injury. The expression of nuclear p65 protein and p50 protein increased compared with that of the control, while the cytoplasmic IκB protein decreased as the inflammation persisted. The expression of COX-2 in CCl_4-treated rat liver increased compared with that of the control. It could be suggested that ROS produced by CCl_4 treatment increased NF-κB binding activity and thereby COX-2 expression, and these might be implicated in the progress of chronic liver damage.

Key Words : *NF-κB; Prostaglandin-Endoperoxide Synthase; Carbon Tetrachloride; Liver Failure, Chronic*

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INTRODUCTION

Although diverse experimental models of liver injury, including hepatic fibrosis and liver cirrhosis, have been established, no model can precisely mimic the real process of human liver disease (1). Even though the results of experiments applied on animals show similar morphology, pathologic processes, and high repetition in comparison with the disease process occurring in the human body, the exact mechanism causing hepatic injury in each experimental model is not yet known. Carbon tetrachloride (CCl_4) has been used most frequently in attempting to establish a liver injury model. The hepatic P450 enzyme system converts CCl_4 into trichloromethyl radical, which causes lipid peroxidation of polyunsaturated fatty acids in cell membranes, and thus induces destruction of cell membranes and cell necrosis (2).

Studies of causative factors involving liver injury have been performed for several years, and now we know that reactive oxygen species (ROS), which are related to oxidative stress, are involved in some types of liver injury (3-6). It is also known that proinflammatory and cytotoxic cytokines play an important role in cell injury of acute fulminant hepatitis (7-10). Even though the relationship between oxidative stress, cytotoxic cytokines, and liver cell injury have not been fully understood, nuclear factor kappa B (NF-κB) is considered to play an important role in liver cell injury.

NF-κB complexes exist in the cytoplasm of most resting cells as an inactivated form of homodimer (e.g., p50-p50) or heterodimer (e.g., p50-p65) bound to a member of the IκB family of inhibitory proteins. It is separated from IκB and is activated by many factors such as tumor necrosis factor, interleukin-1, lipopolysaccharide (LPS), oxidative stress, and human immunodeficiency virus. Activated NF-κB is delivered to the nucleus and is bound to a recognition sequence of DNA consisting of 10 base pairs. It is known that this process increases the release of several cytotoxic cytokines and inflammatory cytotoxins (11-13).

The biosynthesis of prostaglandins (PGs) requires catalytic action of cyclooxygenase (COX) and involves in generation of ROS. COX has been found in two forms, COX-1 and COX-2. The former is a constitutive enzyme which fulfills a role under normal physiologic conditions. This synthesizes various types of PGs in order to maintain human homeostasis. The latter is an inducible isoform which is responsible for production of a large amount of PGs especially when activated by stimuli, such as inflammation, cytokines, and LPS (14-18). ROS is produced by the action of COX-2 which converts PGG2 into PGH2 (19). The synthesis of ROS subsequently activates NF-κB, which has been known to contribute to vicious cycles of inflammatory reactions. It has been

shown that various types of PGs produced by COX-2 involve inflammation of liver cells. The level of COX-2 mRNA and its protein is up-regulated by stimuli of TNF-α. It has been observed that the production of COX-2 mRNA and its protein is inhibited significantly by supplying NF-κB inhibitor, Ad5IKB, to HT-29 cells (20). Thus it is thought that the activation of NF-κB may play an important role in the expression of COX-2.

In this regard, we investigated the NF-κB binding activity and the expression of COX-2 in chronic liver injury induced by CCl_4. CCl_4 was selected because it produces a large amount of PGs especially when activated by stimuli, such as inflammation, cytokines, and LPS. ROS is produced by the action of COX-2, thus we sought to determine whether the activation of NF-κB induced by ROS contributes to liver injury and expression of COX-2 or not.

MATERIALS AND METHODS

Experimental Animal Preparation

After intraperitoneal injection of 0.1 mL of CCl_4/100 g body weight and 0.1 mL of mineral oil/100 g body weight twice a week, 5 Sprague-Dawley rats were sacrificed by decapitation and liver tissues were removed at 1, 3, 5, and 7th week respectively. The removed liver tissues were rinsed in ice-cold buffer (100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethyl-sulfonylfluoride (PMSF), 1 μM pepstatin, 80 mg/L trypsin inhibitor, 2 μM leupeptin, pH 7.4) and immediately immersed in liquid nitrogen and stored at -80°C. The control group was given 0.2 mL of mineral oil/100 g body weight intraperitoneally.

Materials

Chemical reagents were obtained from Sigma, except as noted. The radionucleotide [γ-^{32}]P-ATP was obtained from Amersham (Bucks, U.K.). 2′,7′-dichlorofluorescein diacetate (DCFDA) was from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Anti-COX-1, anti-COX2, anti-p50, anti-p65, anti-IκBα antibodies were obtained from Millipore Corporation (Bedford, MA, U.S.A.). All other materials were obtained in the highest available grade.

Histology

After obtaining liver tissues taken from the rats which were sacrificed at 1, 2, 5, and 7th week. Hematoxylin-eosin and Masson-trichrome stain were applied to those tissues.

Total protein extraction

One gram of liver tissue was added to 10 mL of homogenizing buffer (50 mM potassium phosphate buffer containing 1 mM EDTA, 0.5 mM PMSF, 1 μM pepstatin, 80 mg/L trypsin inhibitor, pH 7.4) and centrifuged at 900 *g* at 4°C for 15 min. The supernatants were re-centrifuged at 12,000 *g* at 4°C for 15 min. The pellets were referred to as mitochondrial fractions and supernatants as postmitochodrial fractions. The supernatants were further centrifuged at 24,000 *g* at 4°C for 1 hr. The pellets were referred to as microsomal fractions and supernatants as cytosol fraction.

Nuclear extract preparation

All solutions, tubes, and centrifuges were maintained at 0-4°C. The preparation of rat liver nuclear extracts was based on the method by Hattori et al. (22). For extracting nuclear proteins from cells, rat liver tissues were treated with inducers including LPS and ROS, and then nuclear extracts and cytosolic fractions were prepared by a modification of the method by Dignam et al. (23). The nuclear extract was frozen at -70°C in aliquots until electrophoretic mobility shift assay (EMSA) was done.

The concentration of total protein in the sample was measured with bicinchoninic acids. The protein assay reagent kit was supplied by Sigma.

Total ROS generation assay

The level of generation of ROS was measured by the method popularized by Thomas et al. (21). Twenty-five μM of 2′, 7′-dichlorofluorescein diacetate (DCFDA) was added to the liver homogenate and then changes in fluorescence were measured at excitation wavelength of 485 nm and emission wavelength of 530 nm for 30 min by using a Fluorescence Plate Reader (BIO-TEK Instruments, Inc., Winooski, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of NF-κB transcription factors in nuclear extracts. For EMSA the specific oligonucleotides were synthesized from Bioneer (Daejeon, Korea). End-labeled T4 polynucleotide kinase was obtained from Promega, and the radionucleotide [γ-^{32}P]-ATP was obtained from Amersham. The oligonucleotide used to detect the DNA-binding activites of NF-κB was as follows: 5′-AGCTTCAGAGGGGATTTCCGAGAGG-3′.

Protein-DNA binding assays were performed with 10 μg of nuclear protein. Because salt can affect the binding activity, the concentration of salt was adjusted to the same level in all samples by adding either a low-salt or a high-salt buffer. Nonspecfic binding was blocked by using 1 μg of poly (dI-dC) · poly (dI-dC). The binding medium contained 4% glycerol and 1.0 mM Tris/HCl (pH 7.5). In each reaction 20,000 c.p.m. of a radiolabeled probe was included. For NF-κB bind-

rabbit polyclonal anti-p65 (Santa-Cruz, 1:200), rabbit polyclonal anti-p50 (Santa-Cruz, 1:200) were also performed. Antibody labeling was detected using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. Pre-stained blue protein markers were used for molecular weight determinations.

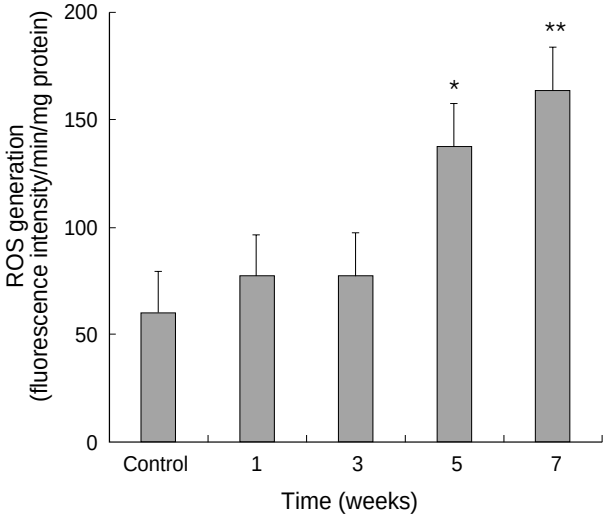

Fig. 2. Effect of CCl_4 on ROS generation in postmitochondrial fraction of rat liver. ROS generation was measured by dichlorofluorescein formation with a fluorescent probe, DCFDA. Each value represents the mean±S.E. from five rats. Statistical significance: *p< 0.05, **p<0.001 vs control rats.

Statistics

Results were analyzed statistically by Krunskal-Wallis test. A p value less than 0.05 was considered statistically significant.

RESULTS

Histology of liver tissue

The liver showed prominent centrilobular fatty change of hepatocytes at 1st week (Fig. 1A) and a more developed pattern at 3rd week (Fig. 1B). There were proliferation of fibrous tissues which surrounded hepatic nodules at 5th week (Fig. 1C) and a more definite cirrhotic pattern at 7th week (Fig. 1D).

Effects of CCl_4 on generation of ROS

The concentration of DCF formed by ROS (fluorescence intensity/min/mg protein) were 76.52±3.20, 77.50±7.68, 137.64±12.12, and 163.20±6.78 at 1, 3, 5 and 7th week, respectively. That of the control group was 60.03±2.68. ROS generation increased significantly in CCl_4-injected groups at 5th (p<0.01) and 7th week (p<0.001), compared to that of the control group (Fig. 2).

Expression of COX-1 and COX-2

Compared to the protein expression of COX in the control group, the protein expression of COX in CCl_4-injected groups showed decreases for COX-1 (p<0.001) (22.66 at control, 21.07 at 1st week, 19.35 at 3rd week, 19.38 at 5th week, 17.77 at 7th week). For COX-2, the CCl_4-injected groups showed statistically significant increase in the protein expression (p<0.001) (5.96 at control, 14.13 at 1st week, 15.48 at 3rd week, 17.28 at 5th week, 13.68 at 7th week), which was

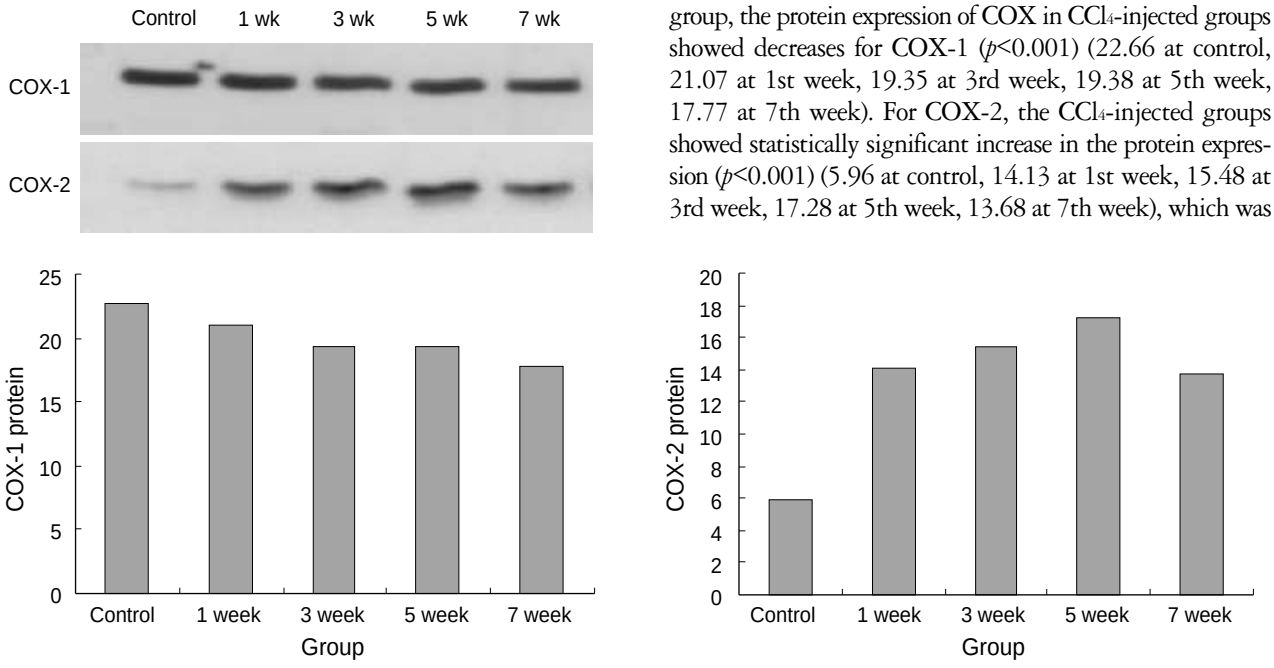

Fig. 3. COX-1 and COX-2 expressions and densitrometric analysis in CCl_4-treated rat liver. Postmitochondrial fractions from control, 1, 3, 5, and 7th week rat tissues were resolved in an 8% SDS-polyacrylamide gel electrophoresis and western blots were performed using anti-COX-1 and anti-COX-2 antibodies. Bands were visualized by ECL procedure (Amersham).

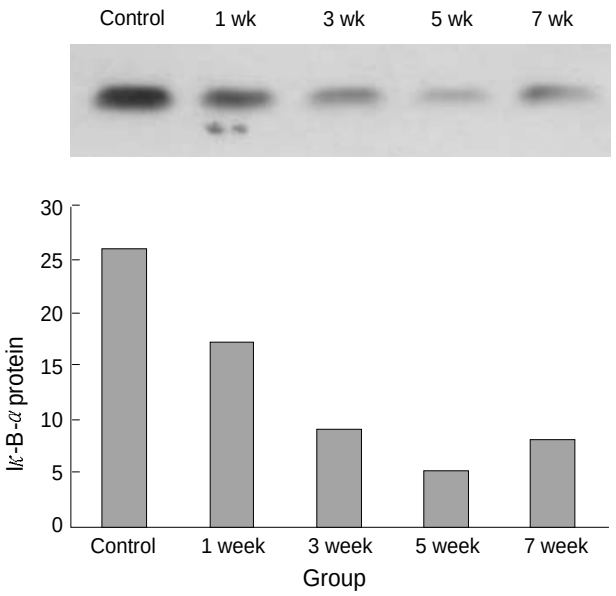

Fig. 6. Cytoplasmic IκB-α protein levels and densitrometric analysis in CCl_4-treated rat liver. Cytoplasmic extracts from control, 1, 3, 5, and 7th week rat tissues were resolved in a 12% SDS-polyacrylamide gel electrophoresis and western blots were performed using anti-IκB antibody. Bands were visualized by ECL procedure.

radical, which forms the trichloromethyl peroxyl radical (6, 24). This ROS, which leads to the process of lipid peroxidation or other mechanisms of cell injury, can induce further interactions. Rats treated with CCl_4 intraperitoneally showed fatty change of liver cells 24 hr after treatment, and severe centriloblular necrosis 24-48 hr after treatment, which resulted from prominent proliferation and ballooning of the endoplasmic reticulum. In this study, the rat liver treated with CCl_4 intraperitoneally also showed typical early extensive centrilobular fatty change and a cirrhotic pattern, which is composed of fibrosis and regenerative nodules several weeks later.

It is known that diverse types of cells and cytokines involved in the inflammation participate in this kind of hepatic injury. These cytokines, as extracelluar stimulants, regulate the genetic expression of various factors and cytokines through the interactions with transcription-modulating factors. It has been considered that a kind of transcription-modulating factor, NF-κB, involves immunologic reactions and apoptosis, and mediates acute inflammatory reactions (25). It was reported that NF-κB plays an important role in the acute liver injury resulted from the action of CCl_4 (26). The previous published studies showed the role of NF-κB in acute inflammatory reactions. It is still not clear whether NF-κB is involved in chronic inflammatory reactions in fibrotic hepatic lesions or liver cirrhosis.

We evaluated the generation of ROS and the changes of NF-κB-binding activity in cases of fibrotic liver and cirrhotic liver caused by CCl_4-induced persistent hepatic inflammation. We observed the increase of the ROS level in the CCl_4-treated group compared to that of the control group. The level of NF-κB-binding activity also increased, and the pattern of the increase was similar to that of ROS. Thus it can be concluded that the activation of NF-κB may play an important role not only in the process of acute liver injury but also in the process of cirrhotic changes.

NF-κB complexes exist in the cytoplasm as a homodimer (e.g., p50-p50) or heterodimer (e.g., p50-p65) bound to a member of the IκB, which mainly acts as an inhibitor of NF-κB. It is known that the NF-κB complexes are activated by phosphorylation and ubiquitination. After the degradation of the IκB component, NF-κB migrates into the nucleus where transactivation is induced by specific DNA binding (27, 28). The signals, which cause the phophorylation and ubiquitination, and follow the degradation of the IκB component, have been known to come from tumor necrosis factor, interleukin-1, lipopolysaccharide, oxidative stress, and human immunodeficiency virus (29).

The western blot analysis of the nuclear proteins revealed an increase in the shifts of p50 and p65 into the nuclei in the groups treated with CCl_4 compared to the control group. The shifts of p50 and p65 into the nuclei tended to increase in proportion to the duration of inflammation. The binding activity of NF-κB is dependent on whether NF-κB is activated or not. Iκ-B-α modulates the activity of NF-κB through mechanisms such as phosphorylation, ubiquitination, and proteasome-mediated degradation.

In this study, the expression of Iκ-B-α decreased in the group treated with CCl_4 compared to the control group. This tended to be proportional to the duration of the inflammation. This suggests that the activation of NF-κB plays an important role not only in the case of acute liver injury but also in the case of chronic persistent liver injury.

The main action of NF-κB in liver injury is to mediate the release of cytotoxic cytokines and inflammatory cytotoxins. Over 100 target genes for NF-κB have been identified during the past few years. One of them is the COX-2 gene. It is known that COX-2 is responsible for inflammatory reaction, carcinogenesis of hepatocellular carcinoma (30, 31), and portal hypertension. Portal hypertension in rats, which was induced by ligation of the portal vein, was improved without gastric mucosal injury by giving the selective COX-2 antagonist NS-398 (32). According to this report, it seems that COX-2 is related to portal hypertension. The overexpressed COX-2, synthesis of which is up-regulated by inflammatory stimuli or hyperoxidation of lipid, induces synthesis of PG, which causes liver injury (33). COX-2 catalyzes the conversion of PGG2 to PGH2, which produces ROS (19). ROS activates NF-κB, which in turn leads to a vicious cycle of inflammation. It has not been made clear what mechanism is involved in the COX-2 expression. It is known, however, that COX-2 is one of the target genes for NF-κB and the Kupffer cells are mainly involved in the expression of COX-2 gene (33).

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