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Carbon tetrachloride-induced hepatic injury through formation of oxidized diacylglycerol and activation of the PKC/NF-κB pathway

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Protein kinase C (PKC) participates in signal transduction, and its overactivation is involved in various types of cell injury. PKC depends on diacylglycerol (DAG) for its activation *in vivo* We have previously reported that DAG peroxides (DAG-O(O)H) activate PKC *in vitro* more strongly than unoxidized DAG, suggesting that DAG-O(O)H, if generated *in vivo* under oxidative stress, would act as an aberrant signal transducer. The present study examined whether DAG-O(O)H are formed in carbon tetrachloride (CCl_4)-induced acute rat liver injury in association with activation of the PKC/nuclear factor (NF)-κB pathway. A single subcutaneous injection of CCl_4 resulted in a marked increase in hepatic DAG-O(O)H content. At the molecular level, immunohistochemistry and subcellular fractionation combined with immunoblotting localized PKCα, βI, βII and δ isoforms to cell membranes, while immunoblotting showed phosphorylation of the p65 subunit of NF-κB, and immunoprecipitation using isoform-specific anti-PKC antibodies revealed specific association of PKCα and p65. In addition, expression of tumor necrosis factor α (TNFα) and neutrophil invasion increased in the CCl_4-treated rats. Furthermore, we demonstrated that Vitamin E, one of the most important natural antioxidants that suppresses peroxidation of membrane lipids, significantly inhibited the CCl_4-induced increase in hepatic DAG-O(O)H content and TNFα expression as well as phosphorylation of PKCα and p65. These data demonstrate for the first time that DAG-O(O)H are generated in the process of CCl_4-induced liver injury, resulting in activation of the PKC/NF-κB pathway and TNFα-mediated aggravation of liver injury.

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Protein kinase C (PKC) has crucial roles in transducing signals that regulate physiological cellular functions, such as cell proliferation, differentiation and apoptosis.[1–3] PKC is also involved in inflammation and cell injury.[4–6] In response to various extracellular stimuli, PKC is activated and triggers the nuclear factor (NF)-κB pathway.[7–9] Select roles of PKC isoforms have been suggested to be involved in alterations of cellular functions as well as activation of the NF-κB pathway. PKC is activated in oxidative stress-related diseases, such as cancer, cerebral ischemia-reperfusion injury and hepatic damage.[10–12] However, the mechanism by which oxidative stress causes the activation of PKC remains unknown.

PKC is activated endogenously by diacylglycerol (DAG) and phosphatidylserine and exogenously by phorbol esters and oxidative stress.[13–15] We have previously reported that DAG peroxides (DAG-O(O)H), which are peroxidation products of DAG, can activate crude rat brain PKC preparations more strongly than unoxidized DAG.[16] We have also revealed that DAG-O(O)H induced superoxide production by human peripheral neutrophils via phosphorylation p47 phox.[17,18] In addition, we have demonstrated that UV irradiation-induced oxidative stress can promote the formation of DAG-O(O)H in the mouse skin.[19] Lipid peroxidation occurs *in vitro* as well as *in vivo* in the presence of reactive oxygen species, such as superoxide,

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hydroxyl radicals and hydrogen peroxide.[20] Oxidative stress resulting from the accumulation of reactive oxygen species has been suggested in cell-signaling alterations.[21] Our previous findings, therefore, indicate that DAG-O(O)H may be generated *in vivo* under oxidative stress and activates PKC excessively.

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin widely used to induce acute toxic liver injury in a wide range of laboratory animals.[22] CCl_4 induces oxidative damage, inflammation, fatty degeneration and fibrosis in the liver.[23–25] NF-κB is activated in the liver after CCl_4 administration and is believed to have a major role in long-term CCl_4 administration-induced chronic liver injury and fibrosis.[26,27]

In the present study, we examined whether DAG-O(O)H are formed in the rat liver in CCl_4-induced acute hepatic injury and activate the PKC/NF-κB pathway.

MATERIALS AND METHODS

Experimental Model

Male Wistar rats (300 g) were injected subcutaneously with an acute hepatotoxic dose of CCl_4 (1.5 ml/kg body weight, Sigma-Aldrich, Dorset, UK). Rats receiving a corn oil alone were used as controls. Animals were humanely killed at 2, 6, 24, 48 or 72 h following CCl_4 administration. In some experiments, Vitamin E (DL-α-tocopherol acetate, 100 mg/ kg body weight, Sigma-Aldrich) was intraperitoneally administered 24 h before CCl_4 administration and animals were killed 6 h after CCl_4 administration. The livers were removed from rats under anesthesia and weighed, snap-frozen in liquid nitrogen and kept at − 80 °C. A portion of the liver was immediately fixed in formalin for histological analyses. Plasma was analyzed for aspartate transaminase activity (SRL, Tokyo, Japan). All animal experiments were approved by the Animal Experimentation Committee, Isehara campus (Tokai University, Kanagawa, Japan).

Immunohistochemistry

A portion of liver tissue was fixed by immersion in Mildform 10 N (Wako, Osaka, Japan) overnight. In all, 4 μm paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded series of ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. For immunostaining of phosphorylated p65 and 4-hydroxy-2-nonenal (HNE), the sections were boiled for 10 min at 98 °C in 10 mM sodium citrate (pH 6.0) to facilitate antigen retrieval. After treatment with 1% normal goat serum blocking buffer for 10 min, the sections were incubated with the primary antibodies, rabbit immunoglobulin G (IgG) against phosphorylated p65 (1:20; Cell Signaling, Beverly, MA, USA) and mouse IgG against HNE (1:20; JAICA, Shizuoka, Japan), overnight at 4 °C. Normal IgG (DAKO, Carpinteria, CA, USA) was used as negative control. For PKC and myeloperoxidase staining, the sections were first autoclaved at 121 °C for 10 min in 10 mM sodium citrate (pH 8.0), and after treatment with 10% normal goat serum blocking buffer for 10 min, the sections were incubated with the primary antibodies, rabbit IgG against PKCα, βI, βII, δ, ε or ζ (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and myeloperoxidase (1:100; Meridian Life Science, Memphis, TN, USA), overnight at 4 °C. Slides were then incubated with the secondary antibody using the Envision HRP Kit (DAKO) for 60 min at room temperature. Phosphorylated p65 staining was then incubated with the secondary antibody for 120 min at room temperature. For TNFα staining, after blocking with 5% rabbit serum for 20 min, the sections were incubated with goat anti-TNFα antibody (1:50; Santa Cruz) for 60 min at room temperature followed by incubation for 30 min with biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min, staining was visualized with 3,3′-diaminobenzidine and nuclei were then counterstained with hematoxylin.

Assay for Oxidized DAGs (DAG-O(O)H)

Assay for DAG-O(O)H was performed as described.[19] Lipids were extracted from liver tissues with 2-propanol containing 1-palmitoyl-3-arachidoylglycerol hydroxide as an internal standard, 20 mM butylated hydroxytoluene and 200 mM triphenylphosphine. Butylated hydroxytoluene and triphenylphosphine were added to prevent artifactual oxidation during the analytical procedure and to reduce hydroperoxide to hydroxide, respectively. The extract was injected into an octadecylsilyl column, and the fraction containing DAG-O(O)H was collected. Methanol was used as a mobile phase. Next, the fraction was injected into a silica column and, using hexane/2-propanol as a mobile phase, the fraction containing DAG-O(O)H was collected. Then, DAG-O(O)H were labeled with pyrene-1-carbonyl cyanide in the presence of quinuclidine, and the labeled DAG-O(O)H were detected by reversed phase HPLC coupled with fluorescence detection (excitation at 330 nm and emission at 405 nm). Various DAG-O(O)H species with different acyl chains were eluted between 35 and 43 min.

Subcellular Fractionation

Subcellular fractionation was performed as described with modifications.[28] Frozen rat liver was homogenized by a Potter homogenizer in 5 ml ice-cold homogenization buffer consisting of 25 mM Tris-HCI (pH 7.4), supplemented with protease inhibitor cocktail (Sigma). The homogenate was first centrifuged at 500 *g* for 5 min at 4 °C to remove tissue debris, then at 100,000 *g* for 60 min at 4 °C. The resultant supernatant containing cytosolic PKC was removed and stored at –80 °C. The pellets were resuspended in 1 ml of the above homogenized buffer containing 1% Triton X-100, and membrane-associated PKC was extracted from the pellets by 5 min of vigorous intermittent vortexing for a total of 30 min on ice. The extract was centrifuged at 100,000 *g* for 60 min,

and the supernatant containing membrane-associated PKC was stored at –80 °C.

Immunoprecipitation

Rat livers were homogenized on ice in lysis buffer consisting of 50 mM Tris-HCl pH 8.0, 150 mM HCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 50 mM NaF and 10 mM sodium pyrophosphate. The lysate was then rotated at 4 °C for 1 h followed by centrifugation at 12,000 *g* for 20 min. The supernatants were combined with 50 µl of protein A sepharose beads (GE Healthcare, Bucks, UK) that had been pre-incubated with anti-PKCα, βI, βII, δ, ε, or ζ antibody (Santa Cruz) and incubated at 4 °C for 90 min. The protein A sepharose beads were spun down and washed thoroughly three times using lysis buffer. The precipitates were resolved on SDS-PAGE gel and subjected to immunoblotting.

Immunoblotting

The protein concentration of each sample was measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were heated at 95 °C for 5 min and applied to 10% SDS-PAGE gels, and then separated proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). After blocking for 60 min at room temperature with 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20, the membranes were incubated overnight at 4 °C with rabbit antibodies against PKCα, βI, βII, δ ε, or ζ (1:800; Santa Cruz), total p65, or phosphorylated p65 (both 1:1000; Cell Signaling) or goat antibodies against TNFα (1:800; Santa Cruz) followed by incubation with peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling) or anti-goat IgG antibody (GE Healthcare), respectively, at room temperature. Immune complexes were visualized by an enhanced ECL detection kit (GE Healthcare).

Statistical Analysis

Values are expressed as means ± SD. Differences were analyzed by Student's *t*-test, and statistical significance was considered when $P<0.05$.

RESULTS

CCl_4-Induced Oxidative Hepatic Injury

To verify hepatic injury, we examined plasma aspartate transaminase activity and histological changes of the liver following CCl_4 administration. Plasma aspartate transaminase activity markedly increased in the CCl_4-treated rats as compared with the control rats (Figure 1a). Histological examination of liver sections at 24 h following CCl_4 administration revealed extensive fatty degeneration of hepatocytes in the centrilobular zone (Figure 1b). HNE is a major end-product of peroxidation of membrane n-6-polyunsaturated fatty acids and is considered a marker of lipid oxidation.[29] To verify oxidative damage, we performed immunohistochemistry for HNE adducts. In the CCl_4-treated rats, HNE adducts were detected in the centrilobular zone as early as 2 h after CCl_4 administration, and their distribution expanded with time (Figures 1c–i).

Quantitative Determination of DAG-O(O)H

Pyrene-fluorescence label techniques were used to investigate the formation of DAG-O(O)H in the CCl_4-treated rats. HPLC analysis of liver homogenates showed that the hepatic DAG-O(O)H content significantly increased with time in the CCl_4-treated rats (Figure 2). It is noteworthy that this increase in the hepatic DAG-O(O)H content was evident as early as 2 h after CCl_4 administration.

Subcellular Localization of PKC Isoforms

Six PKC isoforms are expressed in the rat liver: PKCα, βI, βII, δ, ε and ζ.[30] Immunohistochemistry with isoform-specific anti-PKC antibodies localized intense PKCα expression to the plasma membrane of hepatocytes around the centrilobular vein at 2 and 6 h following CCl_4 administration (Figure 3a). PKCβI, βII and δ were also localized to the membrane of hepatocytes at 2 and 6 h, respectively. Neither PKCε nor PKCζ had translocated to the plasma membrane up to 6 h following CCl_4 administration. Moreover, the subcellular distribution of PKC isoforms was assessed using subcellular fractionation of liver homogenates. Translocation of PKCα, βI, βII and δ to the membrane fraction was seen in the CCl_4-treated rats at 2 and 6 h (Figure 3b). These data were consistent with the immunohistochemical observations. This finding suggests that DAG-O(O)H formed under oxidative stress induced PKC activation.

Phosphorylation of NF-κB p65 in the Liver of CCl_4-Treated Rats

Immunoblotting was used to examine the phosphorylation status of the p65 subunit of NF-κB. Phosphorylated p65 was detected 2 h after CCl_4 administration and increased with time (Figure 4a). Further, it was localized to the nuclei of hepatocytes around the central vein 2 h after CCl_4 administration (Figures 4b and c).

To further determine which PKC isoform phosphorylated p65, immunoprecipitation was performed using anti-PKCα, βI, βII, δ, ε, or ζ antibody, and the precipitates were analyzed by immunoblotting. As shown in Figure 5, PKCα, but not other PKC isoforms, was co-immunoprecipitated with the total p65 from liver homogenates of the CCl_4-treated rats. Interestingly, PKCα was also co-immunoprecipitated with phosphorylated p65. These data suggest that phosphorylation of NF-κB p65 is induced by the PKCα isoform in the liver of CCl_4-treated rats.

TNFα Expression and Neutrophil Invasion in the Liver of CCl_4-Treated Rats

We used immunohistochemistry and immunoblotting to examine the expression of TNFα, one of the targets of NF-κB. TNFα was intensely expressed 2 and 6 h after CCl_4 administration in the cytoplasm of hepatocytes around the

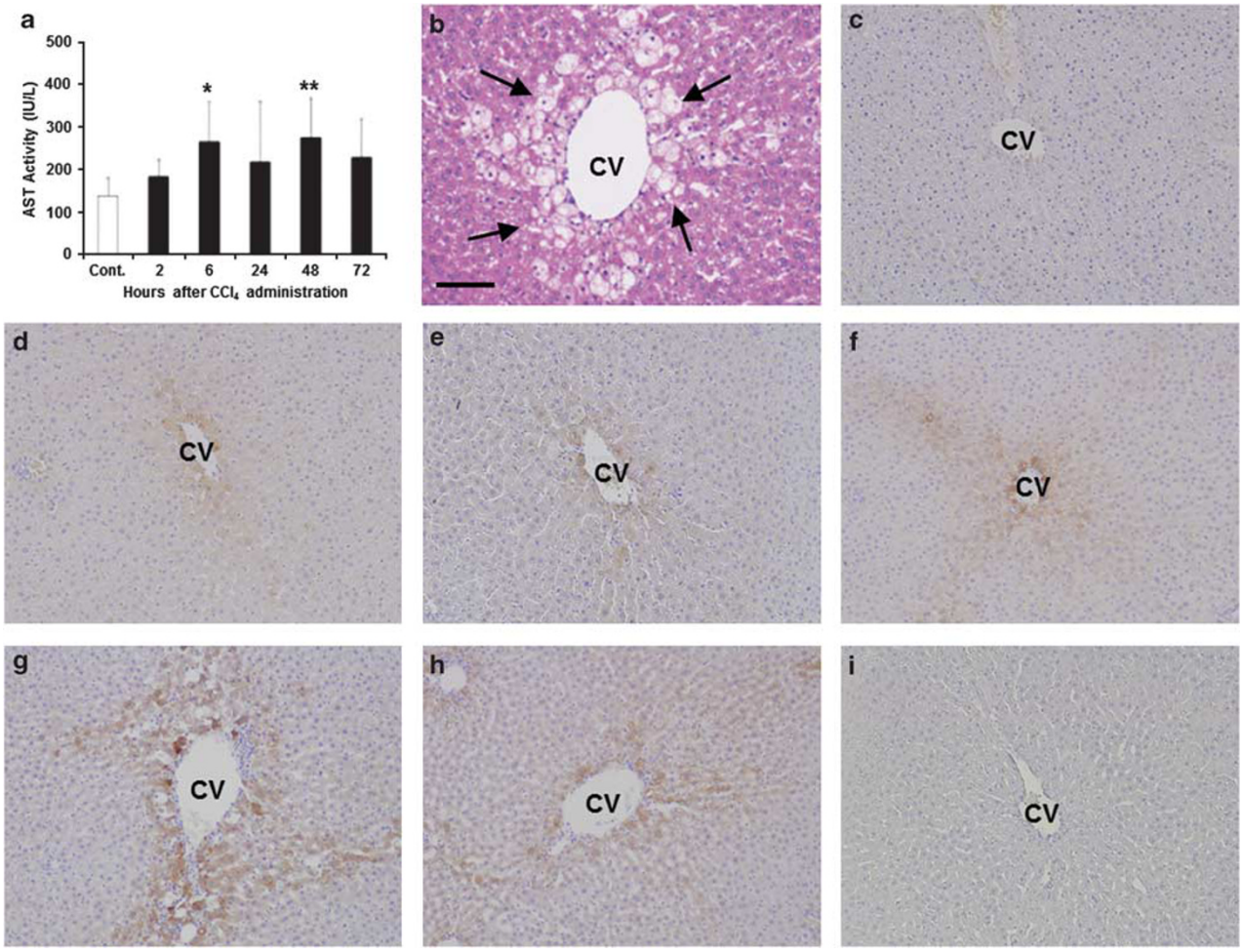


Figure 1 Oxidative liver injury following carbon tetrachloride (CCl_4) administration. (**a**) Plasma aspartate transaminase (AST) activity. Rats were administered vehicle (Cont.) or an acute hepatotoxic dose of CCl_4 (1.5 ml/kg body weight), and plasma was collected 2, 6, 24, 48 and 72 h. Each bar represents the mean ± SD for six rats. $^*P<0.05$ *vs* control, and $^{**}P<0.01$ *vs* control by Student's *t*-test. (**b**) Hematoxylin and eosin staining of liver sections around the centrilobular vein (CV) at 24 h following CCl_4 administration (Bar = 150 μm). Arrows indicate fatty degeneration of hepatocytes. (**c**–**h**) Immunohistochemical localization of 4-hydroxy-2-nonenal (HNE) adducts. Liver sections from (**c**) vehicle-administered control and (**d**–**h**) CCl_4-treated rats were harvested (**d**) 2 h, (**e**) 6 h, (**f**) 24 h, (**g**) 48 h and (**h**) 72 h following CCl_4 administration and stained using anti-HNE antibody. (**i**) Staining control using non-immune mouse IgG.

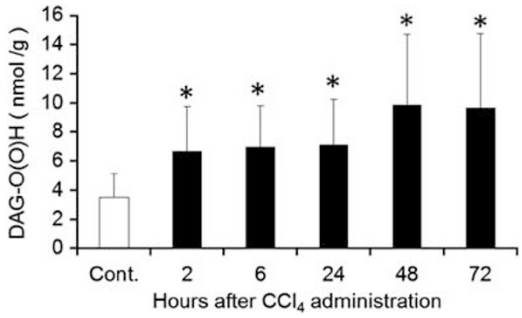


Figure 2 Hepatic diacylglycerol peroxides (DAG-O(O)H) content in carbon tetrachloride (CCl_4)-induced acute liver injury. Livers from vehicle-administered (Cont.) and CCl_4-treated rats were harvested 2, 6, 24, 48 and 72 h after CCl_4 administration, and DAG-O(O)H content was determined as described in Materials and Methods. Each bar represents the mean ± SD for six rats. $^*P<0.05$ *vs* control by Student's *t*-test.

centrilobular vein, but the expression decreased by 24 h (Figure 6). Moreover, we observed immunolocalization of myeloperoxidase, a marker of neutrophils, in the centrilovular zone of the CCl_4-treated rats 2 and 6 h after administration by immunohistochemistry and hematoxylin and eosinstaining (Figure 7). Our data suggest a role for lipid peroxidation-mediated DAG-O(O)H formation in CCl_4-induced expression of cytokines and recruitment of inflammatory cells leading to hepatic injury.

Effects of Vitamin E Administration on CCl_4-Induced Alterations

We administered Vitamin E, one of the most important natural antioxidants that suppress peroxidation of membrane lipids, 24 h before CCl_4 injection, and all animals were killed

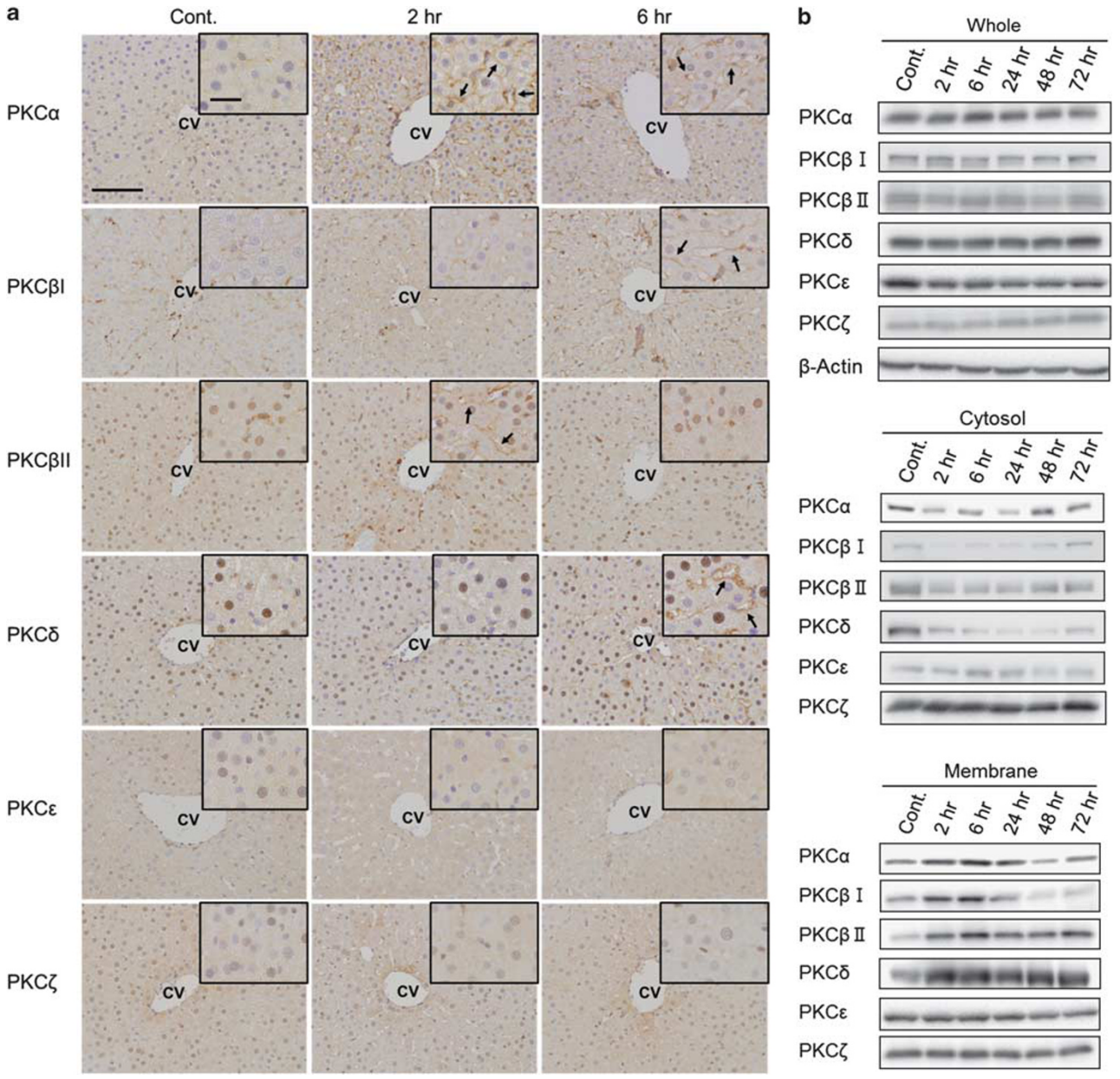


Figure 3 Immunohistochemical localization of protein kinase C (PKC) isoforms in carbon tetrachloride (CCl_4)-induced acute liver injury. (**a**) Liver sections from vehicle-administered (Cont.) and CCl_4-treated rats harvested 2 and 6 h after CCl_4 administration were stained with isoform-specific anti-PKC antibodies. Representative sections are presented (Bar = 100 μm). Insets are enlarged views (Bar = 30 μm). Arrow heads indicate concentrated localization of PKC in the plasma membrane. CV, centrilobular vein. (**b**) The livers from vehicle-administered (Cont.) and CCl_4-treated rats harvested 2, 6, 24, 48 and 72 h after administration were homogenized and fractionated into cytosolic and membrane fractions. Unfractionated homogenate (Whole), cytosolic (Cytosol) and membrane (Membrane) fractions were subjected to immunoblotting using isoform-specific anti-PKC antibodies.

6 h after CCl_4 administration. Vitamin E blocked CCl_4-induced increases in plasma aspartate transaminase activity (Figure 8a), fatty degeneration and HNE adducts and preserved liver histology (Figure 8b). The preserved liver function and histology were associated with suppression of the increase in hepatic DAG-O(O)H content (Figure 8c). Immunoblotting using phosphorylated PKCα/βII antibody showed that Vitamin E significantly diminished phosphorylation of PKCα/βII and NF-κB p65 (Figures 8d and e). Furthermore, Vitamin E significantly inhibited TNFα expression in the rat livers (Figure 8f). These data demonstrate that formation of DAG-O(O)H is involved in inflammatory hepatic injury via the PKC-NF-κB pathway in CCl_4-treated rat liver.

DISCUSSION

This study addressed the question of whether DAG-O(O)H are formed *in vivo* under oxidative stress and are associated

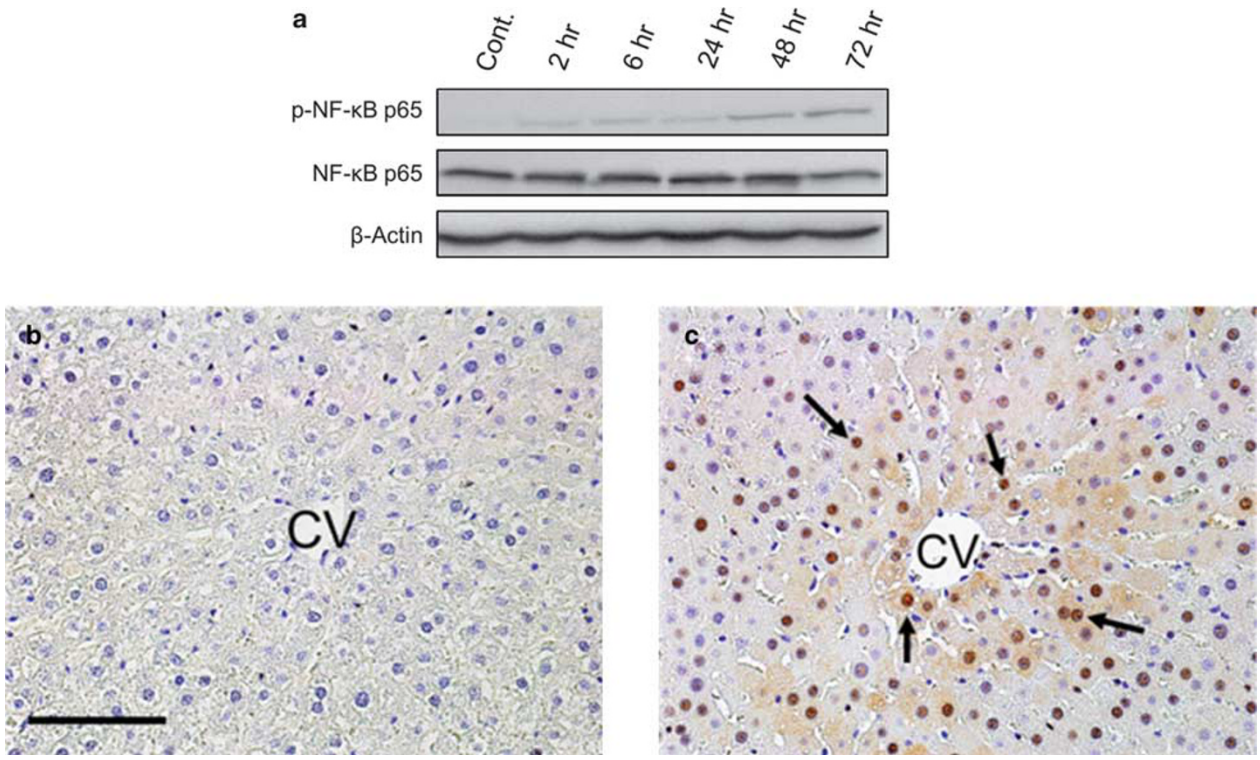


Figure 4 Phosphorylation of the nuclear factor (NF)-κB p65 subunit in carbon tetrachloride (CCl_4)-induced acute liver injury. (**a**) The livers from vehicle-administered (Cont.) and CCl_4-treated rats harvested 2, 6, 24, 48 and 72 h after administration were homogenized and subjected to immunoblotting using antibodies against phosphorylated or non-phosphorylated form of p65 subunit of NF-κB. The β-actin bands were used to correct for protein loading. (**b** and **c**) Immunohistochemical localization of phosphorylated NF-κB p65 subunit. The livers from (**b**) vehicle-administered and (**c**) CCl_4-treated rats harvested 2 h after administration were stained for phosphorylated NF-κB p65 subunit. Arrows indicate intranuclear localization of the phosphorylated NF-κB p65 subunit (Bar = 100 μm). CV, centrilobular vein.

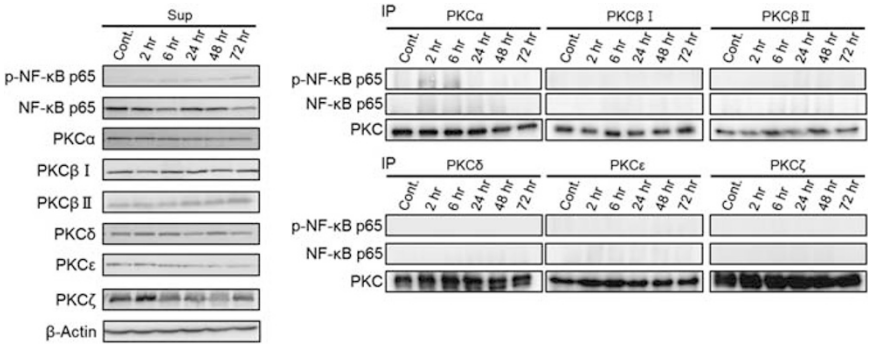


Figure 5 Physical association of protein kinase C α (PKCα) and nuclear factor (NF)-κB p65 subunit. The livers from vehicle-administered (Cont.) and carbon tetrachloride-treated rats harvested 2, 6, 24, 48 and 72 h after administration were homogenized and subjected to immunoprecipitation using isoform-specific anti-PKC antibodies. Left panel, the supernatant fraction (Sup) were analyzed by immunoblotting with antibodies against phosphorylated (p-NF-κB p65), non-phosphorylated NF-κB p65 subunit (NF-κB p65) or PKC isoforms. The β-actin bands were used to correct for protein loading. Right panels, the liver homogenates were immunoprecipitated (IP) with antibodies against PKC isoforms, and the precipitates were analyzed by immunoblotting using phosphorylated (p-NF-κB p65) or non-phosphorylated NF-κB p65 subunit (NF-κB p65) antibodies.

with progression of organ injury. For this purpose, we used a CCl_4-induced rat model of acute liver injury characterized by increased oxidative stress.[31] Our results showed that DAG-O(O)H were formed in the liver upon CCl_4 administration in parallel with accumulation of HNE adducts in the centrilobular zone. Interestingly, some but not all PKC isoforms were translocated to the plasma membrane, and the p65 subunit of the NF-κB complex was phosphorylated and physically associated with PKCα. In addition, TNFα expression was increased at molecular levels. Furthermore, we demonstrated that administration of Vitamin E effectively inhibited the increases in hepatic DAG-O(O)H content and TNFα expression as well as phosphorylation of PKCα and p65 and preserved liver histology. These findings demonstrated for the first time that DAG-O(O)H were formed *in vivo* under oxidative stress and indicated that these

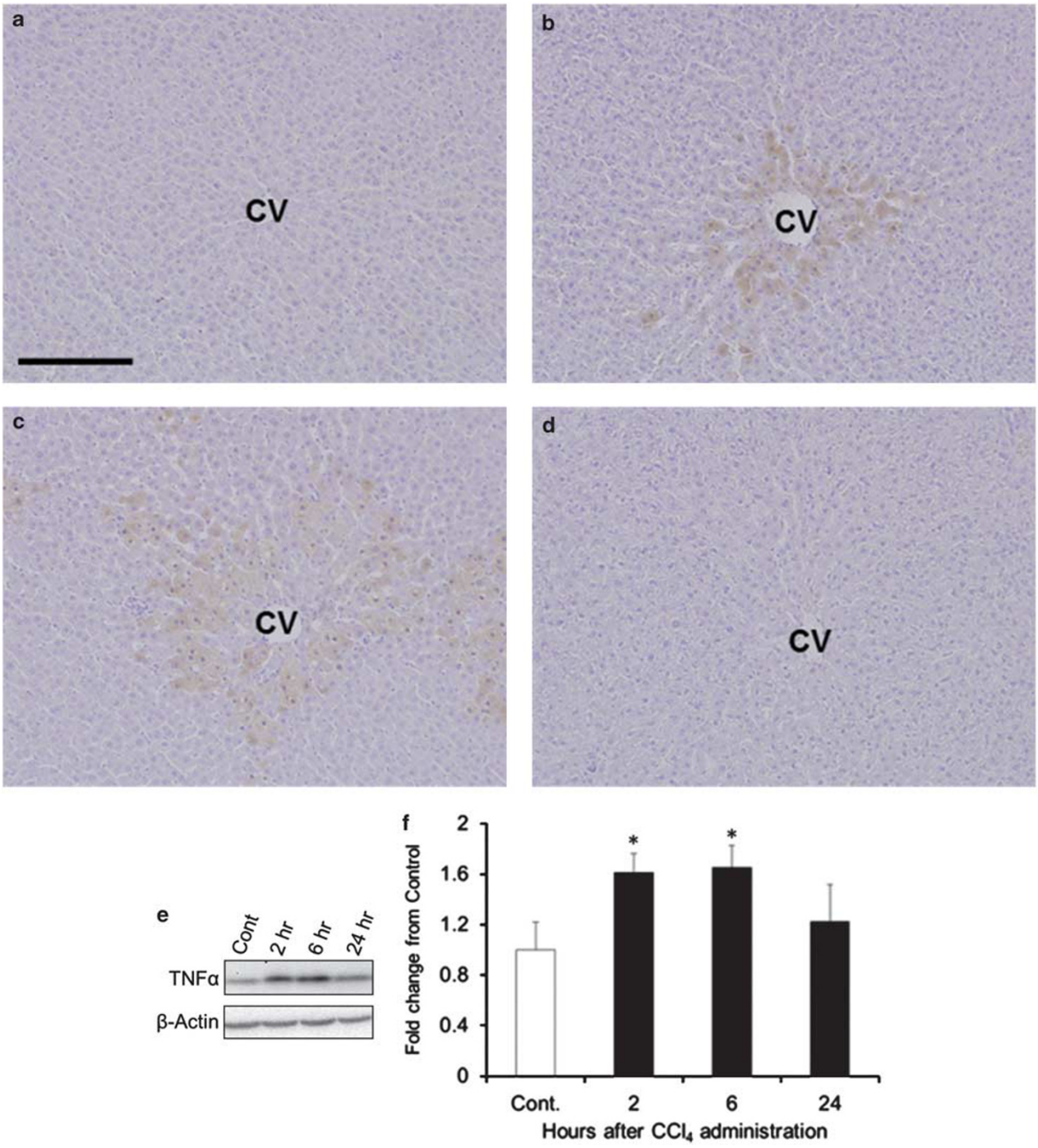


Figure 6 Increased tumor necrosis factor α (TNFα) expression in carbon tetrachloride (CCl_4)-induced acute liver injury. (**a**–**d**) Immunohistochemical localization of TNFα. Liver sections from (**a**) vehicle-administered and (**b**–**d**) CCl_4-treated rats harvested at (**b**) 2 h, (**c**) 6 h and (**d**) 24 h after administration were stained using anti-TNFα antibody. CV, centrilobular vein; (Bar = 200 μm). (**e** and **f**) TNFα expression. TNFα levels were determined by immunoblotting in the livers from vehicle-administered (Cont.) and CCl_4-treated rats harvested 2, 6 and 24 h after administration. The β-actin bands were used to correct for protein loading. Each bar represents the mean ± SD for six rats. $^*P < 0.05$ *vs* control by Student's *t*-test.

molecules activated PKC, leading to the activation of the NF-κB pathway and increased production of inflammatory cytokines.

Upon CCl_4 administration, HNE adducts, which are aldehyde byproducts of lipid peroxidation, were found to be increased in the centrilobular zone by immunohistochemistry as early as 2 h after treatment, thus indicating lipid peroxidation.[32] Coinciding with the increase in HNE adducts, DAG-O(O)H increased in the liver 2 h after CCl_4 administration. These findings indicate that this reagent

caused lipid peroxidation primarily in the centrilobular zone, resulting in the generation of DAG-O(O)H.

Using immunohistochemistry and subcellular fractionation, we found that PKCα, βI, βII and δ were translocated to the plasma membrane in the CCl_4-treated rats. Membrane localization of PKCα, βI, δ and ε has been previously reported in cirrhotic livers of chronically CCl_4-treated rats,[30] but for CCl_4-induced acute liver injury, the present study is the first to demonstrate selective activation of PKCα, βI, βII and δ as evidenced by their membrane localization. PKCα, βI and βII are conventional PKC isoforms that require both DAG and Ca^{2+} ions for activation, while PKC δ and ε are 'novel PKC' that need only DAG.[33,34] Neither DAG nor Ca^{2+} ions are required for activation of atypical PKCζ.[35] Thus, the translocation of conventional PKCα, βI and βII and novel PKC δ isoforms observed in CCl_4-induced acute liver injury suggests release of sequestered Ca^{2+} ions, implicating activation of phospholipase C, findings which corroborate a previous study reporting that CCl_4 administration is associated with PLC activation in rat liver.[36] On the other hand, it has been shown that DAG-O(O)H can activate brain PKC *in vitro* in the absence of Ca^{2+} ions.[16] However, the possibility that DAG-O(O)H alone can activate PKC in CCl_4-induced acute liver injury is unlikely because PKCε was not translocated in our present study.

Immunoprecipitation with isoform-specific anti-PKC antibodies demonstrated physical association of NF-κB p65 with PKCα, but not with PKCβI, βII or δ. These data indicate that despite the activation of PKCα, βI, βII and δ, PKCα alone mediates NF-κB activation with direct molecular interaction in CCl_4-induced acute liver injury, adding another example to selective PKCα isoform involvement in NF-κB activation.[7,8] The observed co-immunoprecipitation of PKCα with both phosphorylated and unphosphorylated forms of p65 suggest continuous, rather than transient, PKCα-mediated NF-κB activation. On the other hand, it has been noted that PKCδ activates NF-κB in oxidative stress condition. Storz *et al.*[37] have demonstrated that PKCδ can activate NF-κB through the

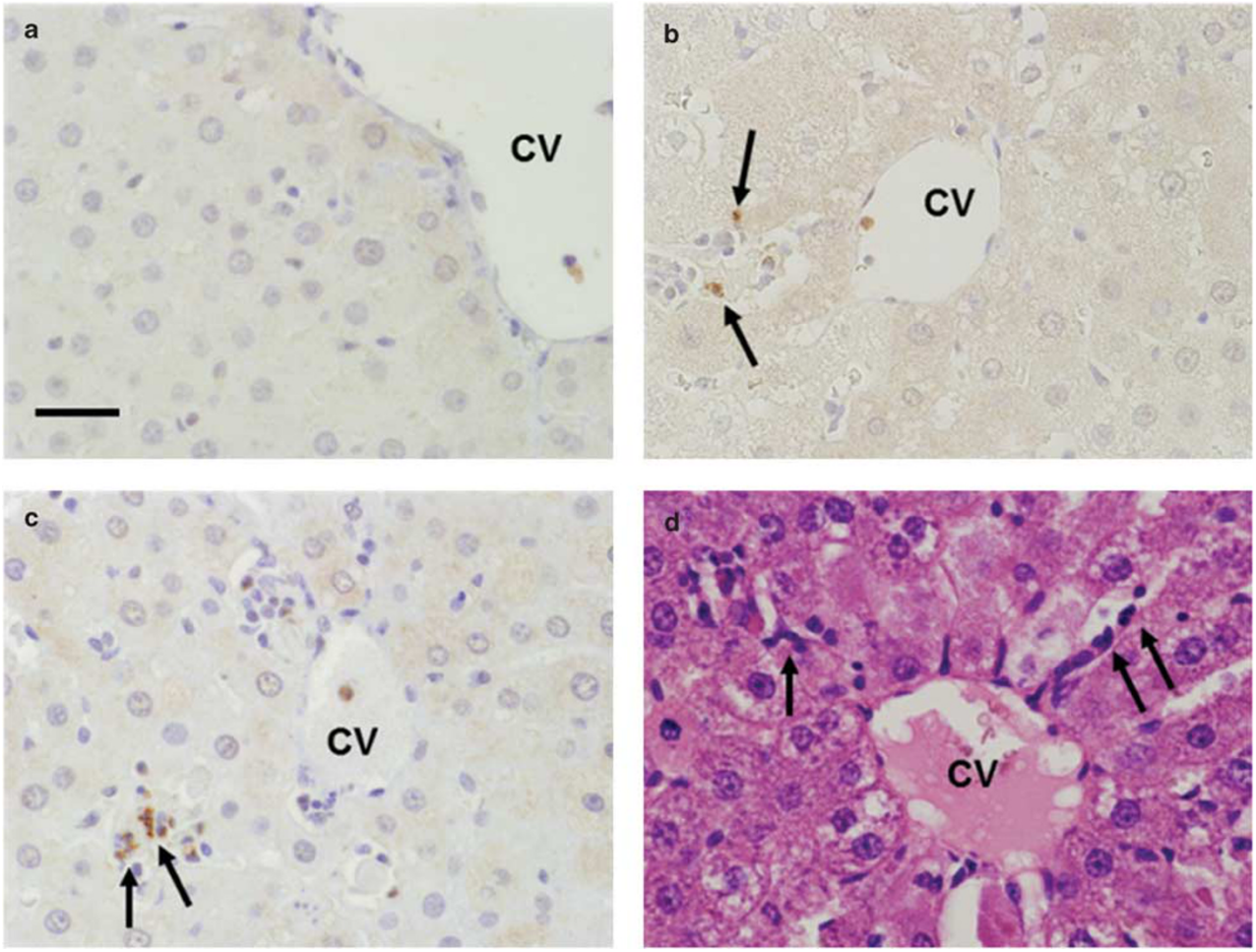
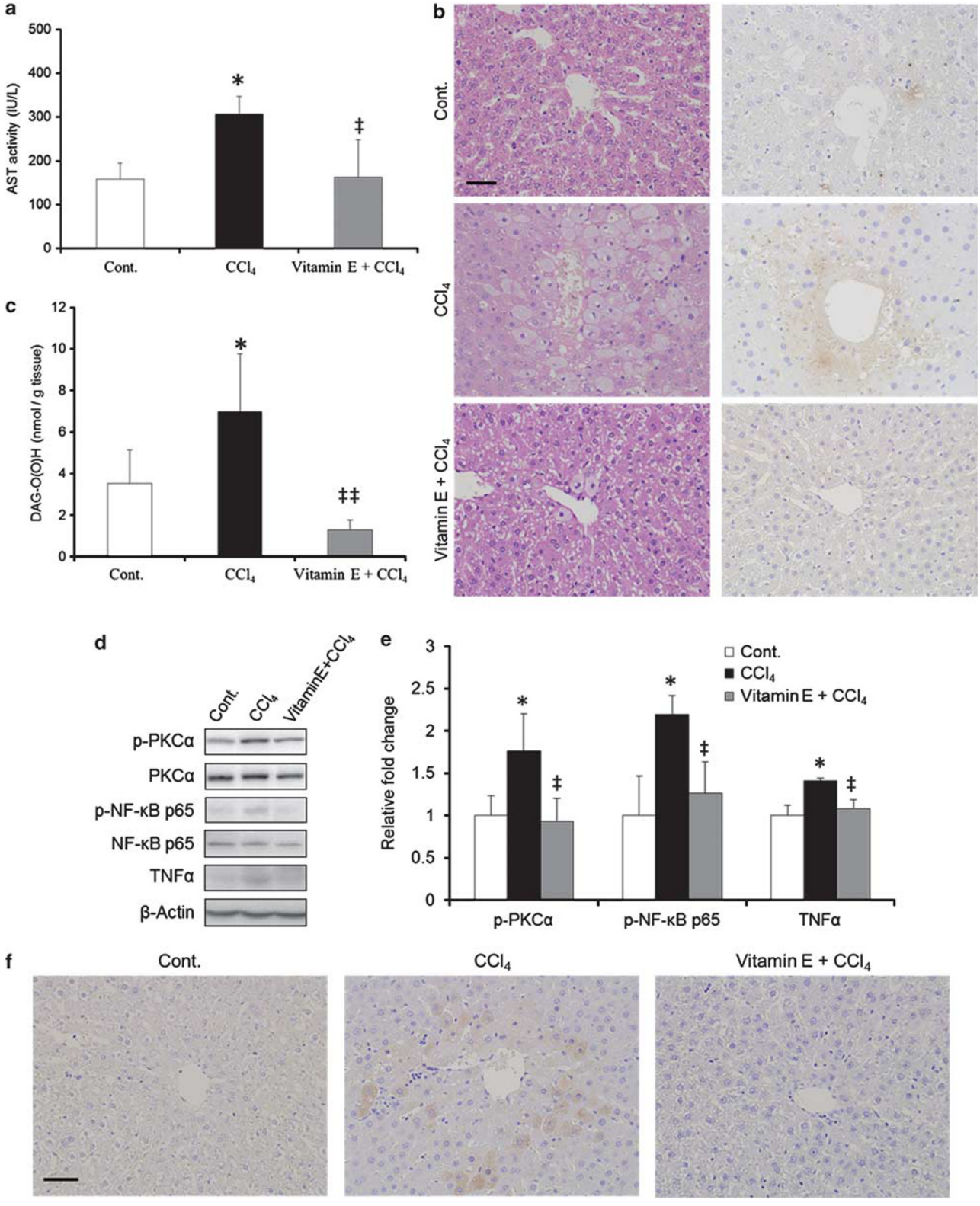


Figure 7 Neutrophil invasion in carbon tetrachloride (CCl_4)-induced acute liver injury. (**a**–**c**) Immunohistochemical localization of myeloperoxidase, marker of neutrophils. Liver sections from (**a**) vehicle-administered and (**b**–**d**) CCl_4-treated rats harvested at (**b**) 2 h and (**c**) 6 h after administration were stained using anti-myeloperoxidase antibody (Bar = 30 μm). (**d**) Hematoxylin and eosin staining of liver sections around centrilobular vein (CV) at 6 h following CCl_4 administration. Arrows indicate neutrophils.

phosphorylation of PKD catalytic kinase domain in cells exposed to oxidative stress. Furthermore, our results indicated that PKCδ is also activated as well as PKCα in CCl_4-treated rat liver. Accordingly, it is conceivable that NF-κB may be indirectly activated by an alternative PKCδ signaling pathway.



Immunohistochemistry and immunoblotting demonstrated increased expression of TNFα, a target of transcriptional activation by NF-κB, further underlining activation of NF-κB in CCl_4-induced acute liver injury.[26,38,39] Several reports have pointed out the importance of inflammation in CCl_4-induced hepatic injury.[40] TNFα knockout mice showed decreased liver injury following CCl_4 administration.[41] On the other hand, decreased expression of TNFα at 24 h was not correlated with time-dependent accumulation of DAG-O(O)H. Previous reports also showed similar findings that expression of TNFα increased in the livers of CCl_4-treated rodents up to 12 h after reagent administration and decreased at 24 h after CCl_4 administration.[41,42] Furthermore, expression of interleukin-10, which is involved in inhibition of TNFα expression is increased 24 h after CCl_4 administration.[43,44] Therefore, decreased expression of TNFα at 24 h after CCl_4 administration is considered to be through interleukin-10 signaling and independent from the PKC-NF-κB pathway.

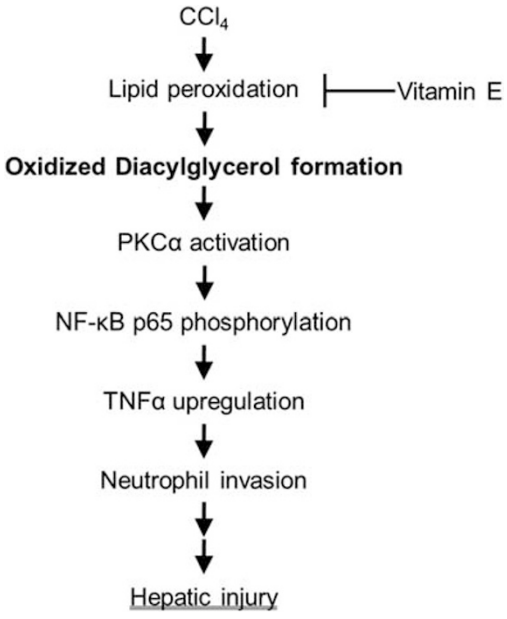


Figure 9 Proposed model of diacylglycerol peroxides (DAG-O(O)H)-induced hepatic injury. DAG-O(O)H induce activation of protein kinase C α (PKCα) and nuclear factor (NF)-κB p65 phosphorylation, which ultimately increases tumor necrosis factor α (TNFα) expression and induces neutrophil invasion. Vitamin E effectively inhibits the formation of DAG-O(O)H content and PKC/NF-κB pathway, with amelioration of liver injury. CCl_4, carbon tetrachloride.

We demonstrated that previous administration of Vitamin E effectively inhibited the formation of DAG-O(O)H *in vivo*. However, membrane translocation of PKC could not be used to determine the consequence of Vitamin E -induced inhibition of DAG-O(O)H formation because it has been reported that Vitamin E directly promotes PKC translocation to plasma membranes, although without activating it,[45,46] and our attempts indeed showed that Vitamin E also induced membrane translocation of PKC (data not shown). We therefore examined PKCα/βII autophosphorylation, the essential event for PKC activation,[47] and found that PKCα/βII autophosphorylation was suppressed in Vitamin E-pretreated rat livers. We also revealed that Vitamin E suppressed phosphorylation of NF-κB p65, TNFα expression and liver injury. These findings indicate that DAG-O(O)H formed upon CCl_4 administration activate PKC, leading to NF-κB activation and TNFα expression.

Long-term CCl_4 administration is a potent inducer of liver fibrosis.[48] It has recently been reported that PKC inhibitors attenuate fibrotic liver injury.[49] In addition, ebselen, a glutathione peroxidase mimetic, has been found to suppress liver fibrosis in rats with CCl_4-induced cirrhosis.[50] We have also reported that ebselen or phospholipid hydroperoxide glutathione peroxidase (PHGPx) reduces DAG-O(O)H to corresponding hydroxides, whereas the conventional cytosolic glutathione peroxidase does not.[51] A recent report demonstrated that overexpression of PHGPx inhibited phosphatidylcholine hydroperoxide-mediated NF-κB phosphorylation in UVA-irradiated human dermal fibroblasts.[52] As UV irradiation-induced oxidative stress can induce the formation of DAG-O(O)H in mouse skin,[19] NF-κB phosphorylation in the above UV-irradiated human dermal fibroblasts may also involve DAG-O(O)H-mediated PKC activation. Long-Evans Cinnamon (LEC) rat is a mutant strain that displays hereditary hepatitis with elevation of copper levels and reactive oxygen species accompanied by severe jaundice at about 3–4 months after birth and develop hepatic cancer at about 1 year after birth.[53] In LEC rats, hepatic DAG-O(O)H levels markedly increased but not in trientine-treated rats, which is a strong copper chelator (data not shown). Consequently, it is conceivable that DAG-O(O)H may participate in the occurrence of chronic liver

Figure 8 Inhibition of diacylglycerol peroxides (DAG-O(O)H) formation by Vitamin E is associated with amelioration of liver injury. (**a**) Plasma aspartate transaminase (AST) activity. Rats were administered vehicle (Cont.) or an acute hepatotoxic dose of carbon tetrachloride (CCl_4) 6 h after administration. A group of rats were intraperitoneally administered Vitamin E 24 h before CCl_4 injection (Vitamin E + CCl_4). Each bar represents the mean ± SD for four rats. $^*P<0.05$ *vs* control, and $^{\ddagger}P<0.05$ *vs* CCl_4 by Student's *t*-test. (**b**) Hematoxylin and eosin staining (left panels) and immunohistochemistry for 4-hydroxy-2-nonenal (HNE) adducts (right panels) of liver sections around the centrilobular vein (CV; Bar = 50 μm). (**c**) Hepatic DAG-O(O)H content. Each bar represents the mean ± SD for six rats. $^*P<0.05$ *vs* control, and $^{\ddagger\ddagger}P<0.01$ *vs* CCl_4 by Student's *t*-test. (**d**) Immunoblotting for protein kinase C α (PKCα)/βII, nuclear factor (NF)-κB p65 and tumor necrosis factor α (TNFα) proteins. Liver homogenates were immunoblotted with antibody against phosphorylated PKCα/βII (p-PKCα), non-phosphorylated PKCα (PKCα), phosphorylated NF-κB p65 (p-NF-κB p65), non-phosphorylated NF-κB p65 (NF-κB p65) or TNFα proteins. The β-actin bands were used to correct for protein loading. (**e**) Densitometric analysis of the immunoblotting shown in (**d**). Each bar represents the mean ± SD for three rats. $^*P<0.05$ *vs* control, and $^{\ddagger}P<0.05$ *vs* CCl_4 by Student's *t*-test. (**f**) Immunohistochemical localization of TNFα in liver sections around the CV (Bar = 50 μm).

disease arising from oxidative stress. We are currently working on this hypothesis.

Taken together, our study demonstrated that DAG-O(O)H are formed in the liver of CCl_4-treated rats and activate PKCα, βI, βII and δ isoforms. We also showed that PKCα activates the NF-κB pathway, leading to increased expression of inflammatory cytokines. Utilizing Vitamin E, we provided evidence that DAG-O(O)H formed *via* lipid peroxidation activate PKC/NF-κB pathway and contribute to the progression of liver injury (Figure 9). We speculate that oxidized DAG underlies aberrant PKC activation seen under oxidative stress.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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