Polarized hepatocytes develop junctional structures that contain adherens junctions (AJs), tight junctions (TJs), and gap junctions. TJs are among the most important functional structures for sealing the bile canalicular apical lumen from the sinusoidal space.1,2 Dysfunction of TJs and distinct biliary transport systems are associated with primary causes of biliary cholestasis and sclerosing cholangitis.1–5 The polarity regulating protein complex, the Par complex, comprises partitioning defective-3 (Par-3), partitioning defective-6 (Par-6), and atypical protein kinase C (aPKC).6,7 Accumulating evidence has established that the Par complex is essential for the maturation of epithelia-specific junctional structures, such as AJs and TJs.6,7 Most polarized cells have a specialized organization of actin and microtubule cytoskeletons that have several roles in cell polarity and the vesicle transport system (vesicle trafficking) because it is regulated by the Par complex.8,9 Knockdown of Par-3 induced a disruption of apical lumen formation and evoked microvillus inclusion bodies that were similar to the phenotype of inflammatory bowel disease and microvillus inclusion disease.8,10 A recent report revealed
that knockdown of Par-3 prevented de novo bile canalculus formation in an in vitro culture system. Selective depletion of aPKCζ in mouse podocytes results in the disassembly of intercellular junctions (slit diaphragms) and focal segmental glomerulosclerosis (FSGS). FSGS pathogenic pathways may involve oxidative stress and inflammation associated with monocellular leukocyte recruitment. However, the molecular mechanisms underlying the roles of the Par complex in oxidative stress and oxidative stress-mediated cell injury remain to be clarified.

We found that carbon tetrachloride (CCl4)−induced oxidative stress resulted in the disassembly of TJs and the translocation of Par-3 and aPKC from TJs to cytoplasm. Defects in the interaction between Par-3 and aPKC lead to the disassembly of junctional structures and also to the loss of Golgi apparatus and orientation in rat livers and were implicated in the disturbance of apical-basal cell polarity. In addition, biochemical analyses revealed that Par-3−aPKC interaction was inhibited by CCl4 treatment. aPKC phosphorylates Ser827 within the aPKC-binding region of Par-3 and thereby down-regulates its own affinity for Par-3. We found that aPKC kinase activity and the phosphorylation level of Ser827 in Par-3 increased significantly. The present results indicated that oxidative stress induced a disturbance of hepatocyte polarity through aberrant activation of aPKC, resulting in the phosphorylation of Ser827 of Par-3 and dissociation of its interaction.

Materials and Methods

Antibodies

All antibodies were purchased from commercial sources as follows: anti−ZO-1, anti−occludin, and anti−claudin-1 rabbit polyclonal antibodies (Life Technologies, Carlsbad, CA); anti−E−cadherin (Clone 36), anti−aPKCζ (Clone 23), and anti−GM130 (Clone 14) mouse monoclonal antibodies (BD Biosciences, San Jose, CA); anti−aPKC (C20) and anti−β−catenin (H-102) rabbit polyclonal antibody, anti−ZO-1 (R40.76) rat monoclonal antibody, anti−TGN38 (B-6), and anti−γ−actin mouse monoclonal antibody (AC15; Sigma-Aldrich). Rats receiving corn oil alone were used as controls. Animals were euthanized at 2 and 6 hours after CCl4 administration (n = 7). In some experiments, i.p. vitamin E (DL-α-tocopherol acetate, 100 mg/kg body weight, Sigma-Aldrich) was administered 24 hours before CCl4 administration, and animals were euthanized 6 hours after CCl4 (n = 3). The livers were removed from anesthetized rats and weighed, snap-frozen in liquid nitrogen, and kept at −80°C for biochemical analysis. A portion of the liver was immediately fixed in formalin for histologic analyses. All animal experiments were conducted in accordance with the guidelines for proper conduct of animal experiments (Science Council of Japan), and all protocols were approved by our institutional review board.

Histologic Analysis and Immunofluorescence Microscopy

Ten percent formalin-fixed, paraffin-embedded liver sections were used for hematoxylin and eosin staining, and 4% paraformaldehyde-fixed frozen sections were used for Oil red O staining. A portion of liver tissue was fixed by immersion in Mildform 10N (Wako Pure Chemical Industries, Ltd., Osaka, Japan) overnight. Paraffin-embedded sections (4 μm) 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 minutes. For immunostaining, sections were autoclaved at 121°C for 15 minutes in 1 mmol/L EDTA (pH 8.0). After blocking with 10% calf serum, the sections were incubated overnight at 4°C with primary antibodies. The secondary antibodies used were Alexa Fluor 488−, 594−, or 647−conjugated goat antibodies against rabbit, mouse, or rat IgG. Cell nuclei were stained with TOPRO3. After immunostaining, the sections were removed from the phosphate-buffered saline wash, dipped briefly in distilled water, treated with copper(II) sulfate (Sigma-Aldrich) in ammonium acetate buffer (50 mmol/L CH3COONH4, pH 5.0) for 2 hours, redipped briefly in distilled water, and returned to phosphate-buffered saline. These sections were mounted with phosphate-buffered saline that contained 90% (wt/vol) glycerol and 0.0125% (wt/vol) DABCO (Sigma-Aldrich) and examined using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Jena, Germany).

Immunohistochemistry

Antibodies were from the following sources and used as indicated: anti-HNE, anti−ZO-1, anti−Par-3, anti−TGN38,
and anti-P-glycoprotein. Slides were treated with 100% methanol and 3% H₂O₂ at room temperature for 30 minutes to reduce endogenous peroxidase activity. The samples were rinsed in water and subjected to antigen retrieval at 121°C for 10 minutes in 10 mmol/L Tris-HCl (pH 10.0) for P-glycoprotein. To stain ZO-1, Par-3, and TGN38, the sections were autoclaved at 121°C for 10 minutes in 1 mmol/L EDTA (pH 8.0). For HNE, sections were autoclaved at 121°C for 10 minutes in 10 mmol/L citrate buffer (pH 6.0). After blocking for 30 minutes with 5% normal calf serum, the sections were incubated overnight at 4°C with primary antibodies. The labeled secondary antibodies were visualized using a HistoFine kit (Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine reaction. Finally, the sections were counterstained with hematoxylin and observed by AX71 microscopy (Olympus, Tokyo, Japan).

Immunoprecipitation and in Vitro Protein Kinase Assays

Rat livers were suspended in lysis buffer and were subjected to immunoprecipitation with 1.5 μg of antibody. For in vitro kinase assay, aPKC was immunoprecipitated with anti-aPKC (C20) antibody. The immunoprecipitates in 40 μL of phosphorylation buffer were pre-incubated with 2 μg of glutathione S-transferase fused to amino acids 633 to 666 of human Llg12 purified protein for 20 minutes on ice. The phosphorylation reaction was started by adding a substrate mix that contained 40 μmol/L ATP. After 30 minutes at 30°C, the reaction was stopped by adding SDS sample buffer. The products were subjected to SDS-PAGE, followed by Western blotting as described previously.15

Figure 1 Carbon tetrachloride (CCL₄)-mediated oxidative stress results in a disturbance of the distribution of junctional complex proteins in rat hepatocytes. A: Rat livers stained with hematoxylin and eosin at control (0 hours), 2 hours, and 6 hours after CCL₄ treatment. B: Control or CCL₄-treated (2 and 6 hours) livers were immunostained for anti-4-hydroxy-2-nonenal (HNE) (red), ZO-1 (green), and nuclei (blue). Arrowheads indicate HNE-positive cells. C: Livers were fixed and stained for β-catenin (red) and ZO-1 (green). Arrows indicate disrupted β-catenin (red) with ZO-1 (green) signal at cell-cell contact sites. D: Western blot data reveal the expression levels of the TJ proteins ZO-1 and claudin-1, the adherens junctions and cytoskeletal proteins E-cadherin and β-catenin, the polarity protein atypical protein kinase C (aPKC), and the internal controls β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). E: Livers were immunostained for claudin-1 (red) (left) or occludin (red) (right) and ZO-1 (green). Confocal stack images are shown in B, C, and E. Scale bars: 50 μm (A); 20 μm (B, C, and E). CV, centrilobular vein; PV, portal vein.
Electron Microscopy

Rat livers were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight at 4°C and then post-fixed with 1% OsO4 in the same buffer for 90 minutes at room temperature (n = 7). The samples were rinsed with distilled water, stained with 0.5% aqueous uranyl acetate, and dehydrated with ethanol. In all, 60- to 90-nm ultrathin sections were examined with a JEM1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV. Ultrathin sections (60 to 90 nm) were cut from 2 to 5 tissue blocks per animal, and the canalicular lumen was measured on >30 sections from each animal.

Statistical Analysis

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

Results

Oxidative Stress Induces Disruption of Cell-Cell Junctions in Hepatocytes

CCL4 induces a phenotype similar to hepatic steatosis and cirrhosis in the liver. The levels of lipid peroxidation are increased in CCL4-treated rat liver.17,18 Fatty degeneration was observed in CCL4-treated hepatocytes facing central veins but not in perportal areas (Figure 1A and Supplemental Figure S1). To elucidate the effect of CCL4 on junctional structures in hepatocytes, we analyzed TJs and AJs at 2 and 6 hours after CCL4 treatment. The centrilobular region of the liver is most susceptible to chemical toxins that are catalyzed by cytochrome P450.18,19 The TJ components, ZO-1, claudin-1, and occludin, were localized in the vicinity of bile canaliculi, where TJ structures were linearly stained in normal rat liver (Figure 1). In contrast, at 6 hours after CCL4 treatment of livers, the localization of these proteins had markedly disappeared from TJ structures (Figure 1, B, C, and E). These defects were observed even at 2 hours of CCL4 administration, but the effects were stronger at 6 hours of CCL4 treatment (Figure 1B and Supplemental Figure S2). On the other hand, the AJ component, β-catenin, was distributed at cell-cell contact sites by CCL4 treatment (Figure 1C). Both TJ and AJ protein expression levels were not changed by CCL4 treatment (Figure 1D). HNE, one of the major final products of lipid peroxidation, has established cytotoxicity.20 Double-label immunofluorescence analysis revealed that HNE signals were observed in ZO-1-negative hepatocytes in CCL4-treated rats (Figure 1B and Supplemental Figure S1A). However, in the CCL4-treated rats, HNE was not detected in the portal area (Supplemental Figure S1).

Most human cholestatic liver disorders are associated with profound deterioration of the barrier function of TJs.1-4 Ultrastructural analysis revealed that TJs were restricted to a narrow band of membrane on either side of a bile capillary (Figure 2A). In contrast, CCL4-treated hepatocytes had disrupted contact points of TJs and expansion of the intercellular space (Figure 2A). Similar data were reported, revealing that CCL4 treatment resulted in the progressive loosening and fragmentation of the junctional meshwork.17 Furthermore, the bile canalicular lumen size, but not the junctional structure length (data not shown), was expanded in CCL4-treated rat livers (Figure 2). The lumen expansion was significantly different between control (0 hours) and after 2 or 6 hours of CCL4 treatment. Data are expressed as means ± SD. n = 38 lumens at 0 hours, n = 14 lumens at 2 hours, and n = 24 lumens at 6 hours. **P < 0.01, ***P < 0.005 versus C by Welch’s t-test. Scale bar = 1 µm.
6 hours, 2.13 ± 1.07 μm) (Figure 2B). These data suggest that CCl₄-induced oxidative stress caused a disturbance of hepatocyte junctional structures and expansion of the bile canaliculi lumen.

Oxidative Stress—Induced Hepatotoxicity Results in Disturbance of Cell Polarity

The structures of the endoplasmic reticulum, plasma membrane, and Golgi apparatus of the liver parenchymal cell are strikingly altered after administration of a single oral dose of carbon tetrachloride to rats. An increased number of unattached ribosomes were found amid altered granular endoplasmic reticulum in CCl₄-treated hepatocytes (Figure 3A). Furthermore, the Golgi apparatus was irregularly dilated in CCl₄-treated cells of the centrilobular zone, and swollen Golgi vacuoles were observed (Figure 3A). These changes, first seen in cells of the centrilobular zone within 2 hours after CCl₄ administration, were progressive to 6 hours (Figure 2A). The Golgi marker, GM130, was asymmetrically localized above the luminal side of the nuclei adjacent to ZO-1—positive cell-cell junctions (Figure 3B). In contrast, disassembly of Golgi bodies was observed in CCl₄-treated hepatocytes (Figure 3B). Furthermore, staining for TGN38, a trans-Golgi network—specific protein, was significantly decreased in the centrilobular zone of CCl₄-treated rats (Supplemental Figure S3A). The Golgi apparatus also serves as a station for protein sorting and lipid transport. A marked increase in lipid accumulation was observed in CCl₄-treated rat livers in the centrilobular zone (Figure 3C and Supplemental Figure S1B). In the liver, P-glycoprotein (MDR1), the product of a multidrug-resistance gene (ABCB1), is found exclusively on the biliary canalicular front of hepatocytes and on the apical surface of epithelial cells. Suplemental Figure S3B shows P-glycoprotein staining on the apical surface of hepatocytes in control rats. Consistent with the apical microvilli formation data (Figures 2 and 3), no significant difference was found between control and CCl₄-treated rats. However, both bile canalicular lumen morphology (size) and Golgi orientation were disturbed by CCl₄ treatment. These results provided support that CCl₄ treatment induced impairment of vesicle transport and apico-basal cell polarity in rat hepatocytes.

Figure 3  Carbon tetrachloride (CCl₄) treatment induces a disturbance of Golgi morphology. A: Transmission electron micrographs show the cytoplasmic matrix of hepatocytes after administration of CCl₄ to an animal. The membrane of the granular endoplasmic reticulum is sparsely and irregularly lined by ribosomes. Furthermore, components of the Golgi apparatus (G) are irregularly swollen (arrowheads), and the number of dilated granules (arrows) increases. BC indicates bile canalculus. B: Rat livers were immunostained for GM130 (red), ZO-1 (green), and nuclei (blue). In control (0 hours) livers, the cis-Golgi marker GM130 is asymmetrically distributed above the bile canalculus side of the nuclei but not in CCl₄-treated livers (6 hours) (arrowheads). Confocal stack images are presented. Right panels are IMAEIS images (Carl Zeiss). CV indicates centrilobular vein. C: Frozen sections of livers were fixed and stained for lipid by oil red O staining. CCl₄-treated liver (2 and 6 hours) sections reveal extensive fatty degeneration of hepatocytes in the centrilobular zone (red signals). Scale bars and grid scale: 2 μm (A); 20 μm (B); 50 μm (C). BC, bile canalculus; CV, centrilobular vein; PV, portal vein.
Interaction between Par-3 and aPKC Is Required for the Maintenance of Hepatocyte Polarity

The Par complex plays a crucial role in TJ formation during epithelial cell polarization and epithelial tissue morphogenesis. We found that Par-3 and aPKC localized to ZO-1-positive cell-cell junctional regions in control rat livers (Figure 4, A and B). These localizations strikingly changed to the cytoplasm in the centrilobular zone after CCl4 treatment (Figure 4A). However, the localization of ZO-1, Par-3, and aPKC was not affected in the portal area of hepatocytes after CCl4 administration. The amount of cytoplasmic co-staining of Par-3 and aPKC was significantly increased in the centrilobular zone at 6 hours after CCl4 administration (control, 22.6 ± 9.4; 2 hours, 36.9 ± 28.2; 6 hours, 56.7 ± 14.5) (Figure 4, A and C). To examine whether the interaction between aPKC and Par-3 is required for maintaining hepatocyte junction and polarity, we analyzed the binding ability of Par-3 to aPKC in CCl4-treated rat liver by immunoprecipitation assay. Using a co-sedimentation analysis, we found that binding of Par-3 to aPKC was severely impaired in CCl4-treated rat livers (Figure 4, D and E). These data indicated that cytoplasmic Par-3 did not bind to aPKC (Figure 4A). Our findings suggested that the interaction between aPKC and Par-3 was necessary to maintain localization and hepatocyte junctional structures.

Oxidative Stress Inhibits Interaction between Par-3 and aPKC through Aberrant aPKC Activation

It was previously reported that aPKC phosphorylates Ser827 on Par-3, which reduces the stable interaction of Par-3 with aPKC. It was previously reported that aPKC phosphorylates Ser827 on Par-3, which reduces the stable interaction of Par-3 with aPKC.
Phosphorylation of Par-3 was enhanced approximately 2.5-fold within 6 hours after CCl₄ treatment (control: 1.00 ± 0.26; 2 hours: 2.12 ± 0.89; 6 hours: 2.22 ± 0.15) (Figure 5, A and B). PI3-kinase produces phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P₃], and the latter activates phosphoinositide-dependent protein kinase-1 (PDK1), which potently activates aPKC and Akt.²⁵,²⁶ PDK1 phosphorylates aPKCζ/λ on Thr410/403 and activates it. We found that the phosphorylation level of aPKCζ/λ increased with CCl₄ treatment (control: 1.00 ± 0.08; 2 hours: 1.57 ± 0.26; 6 hours: 1.56 ± 0.31) (Figure 5, C and D). Furthermore, both Akt-Thr308 and Akt-Ser473 phosphorylations were significantly elevated in CCl₄-treated liver (control, 1.00 ± 0.26; 2 hours, 1.60 ± 0.59; 6 hours, 2.06 ± 0.53) (Figure 5, C and D). The PI3-kinase signaling pathway is activated under oxidative stress.²⁷ Our data support the hypothesis that aberrant activation of aPKC is induced by oxidative stress—mediated activation of PI3-kinase signaling. However, the activation of aPKC in CCl₄-treated rats has not been clarified. A glutathione S-transferase fusion protein with amino acid sequence 636 to 666 of human Llgl2 (Llgl2 peptide) is phosphorylated by purified aPKC from cultured cells in vitro.¹⁵ After CCl₄ treatment, liver lysates were subjected to immunoprecipitation with antibodies against indicated proteins, and the phosphorylation levels of aPKCζ/λ Thr410/403, Akt-Thr308, and Akt-Ser473 were quantified and found to be significantly increased in CCl₄-treated rat liver. E: Con. (0 hours) and CCl₄-treated (2 and 6 hours) rat livers were subjected to immunoprecipitation (IP) with the anti-aPKC antibody or control IgG. The IPs were incubated in the presence of glutathione S-transferase—Llgl2 peptide with ATP and detected by using anti-phospho-Llgl2 Ser653 antibody together with anti-aPKCζ/λ antibody. F: Quantification of the results in E. Phosphorylation of Llgl2 is significantly increased at 6 hours after CCl₄ treatment. Data are presented as means ± SD (B, D, and F). n = 4 (B); n = 3 to 5 (D and F). *P < 0.05, **P < 0.01 versus Con. by Student’s t-test.
Vitamin E Prohibits CCl4-Induced Par-3—aPKC Dissociation and aPKC Activation

Vitamin E is one of the most important natural antioxidants that suppress the peroxidation of membrane lipids by CCl4. In CCl4-treated rat liver, Par-3 and aPKC were delocalized to the cytoplasm from ZO-1-positive cell-cell junctions (Figure 4A and Figure 6A). Vitamin E prevented HNE accumulation by CCl4 treatment (Figure 6A). Moreover, vitamin E prohibited both CCl4-induced disruption of ZO-1 and Par-3 cytoplasmic relocalization (Figure 6A). In addition, we analyzed the interaction between Par-3 and aPKC in rat liver with both CCl4 and vitamin E treatments. Immunoprecipitation analysis revealed that vitamin E significantly prohibited CCl4-induced dissociation of Par-3—aPKC binding (control, 1.00 ± 0.19; CCl4, 0.68 ± 0.14; CCl4 + vitamin E, 1.12 ± 0.23) (Figure 6, B and C). These data suggest that the inhibition of the polarity-regulating Par complex is involved in hepatic injury through aberrant aPKC activation by CCl4-mediated oxidative stress in rat liver.

Discussion

We found that CCl4-induced oxidative stress results in the disassembly of junctional structures. CCl4 induces a phenotype similar to hepatic cirrhosis and cholestasis in rat liver. In several clinical forms of cholestasis and in cholestatic models, distinct functional and morphologic alterations of TJs have been described in hepatocytes. The functional integrity of TJs is crucial in preventing paracellular leakage of bile constituents. However, the molecular mechanisms of disturbance of TJs remain obscure. The Par-3—aPKC complex plays a crucial role in TJ formation during epithelial cell polarization and epithelial tissue morphogenesis. We revealed that the localization of Par-3 and aPKC to the cell junctional region was inhibited by CCl4 treatment. In addition, the interaction between Par-3 and aPKC in rat liver with both CCl4 and vitamin E treatments. Immunoprecipitation analysis revealed that vitamin E significantly prohibited CCl4-induced dissociation of Par-3—aPKC binding (control, 1.00 ± 0.19; CCl4, 0.68 ± 0.14; CCl4 + vitamin E, 1.12 ± 0.23) (Figure 6, B and C). These data suggest that the inhibition of the polarity-regulating Par complex is involved in hepatic injury through aberrant aPKC activation by CCl4-mediated oxidative stress in rat liver.

Figure 6 Vitamin E prevents carbon tetrachloride (CCl4)—induced disassembly of tight junctions (TJs) and Par-3—ataypical protein kinase C (aPKC) interaction. A: Control and CCl4-treated rat livers with (CCl4+VE) or without (CCl4) vitamin E were immunostained for anti-4-hydroxy-2-nonenal (HNE), ZO-1, and Par-3. HNE staining decreases in vitamin E-treated livers. In addition, vitamin E partially prevents CCl4-induced disassembly of ZO-1 and reduces Par-3 staining. B: Livers from vehicle-administered and CCl4-treated rats with or without vitamin E were harvested 6 hours after administration, supernatant fraction (input), and subjected to immunoprecipitation (IP) using antibody against Par-3. Precipitated proteins were analyzed by Western blot. C: Quantification of the amount of aPKC/α in the IP fraction shows significant decrease with CCl4 treatment (CCl4), but not with vitamin E injection (CCl4+VE). Data are presented as means ± SD. n = 3 to 4. *P < 0.05 versus control by Student’s t-test. Scale bar = 40 μm. IP, immunoprecipitation.

CCl4 Induces Excessive aPKC Activation
resulting in the retention of apical transporters on the basolateral surface of hepatocytes and a delay in vesicle transport to the bile canaliculi.\(^2,3,4\) We also found that CCl\(_4\) treatment in rat resulted in Golgi disorientation and disassembly of the trans-Golgi network (Figure 3 and Supplemental Figure S3). In addition, ultrastructural analysis revealed that the Golgi apparatus underwent irregular expansion or tabulation (Figure 3). This altered structure is similar to labyrinthine tubular aggregates but differs from smooth endoplasmic reticulum.\(^3,7\) CCl\(_4\) is converted to a tri-chloromethyl (CCl\(_3\)) radical by the cytochrome P450 system. This radical is very reactive and quenched by adjacent lipids, resulting in lipid peroxidation or binding covalently to membrane proteins and lipids.\(^3,8,9\) In general, the CCl\(_3\) radical reacts with polysomes, causing their disaggregation, and with both endoplasmic reticulum membrane and plasmalemma, distorting their functions and structures.\(^3,7,9\) CCl\(_4\)-induced disturbance of hepatocyte polarity can be caused by altered Golgi structure or disorganized vesicle transport. The orientation of the centrosome contributes to overall cell polarity by positioning both the Golgi and the endocytic recycling compartment. Par-3 is required for centrosome orientation and centration by regulating microtubule dynamics.\(^4,0\) Moreover, Par-3 associates with dynein and a small amount of aPKC. aPKC also interacts with dynein to maintain the microtubule-organizing center at the cell centroid.\(^4,1\) These results suggested that the interaction between Par-3 and aPKC may be necessary for regulating cell polarity through Golgi orientation or other mechanisms (Figure 7).

We revealed that localization of the cell polarity-regulating proteins Par-3 and aPKC to the cell junctional region was inhibited by CCl\(_4\) treatment. Importantly, biochemical analysis revealed that the Par-3—aPKC complex was markedly decreased in CCl\(_4\)-treated rat livers. Furthermore, phosphorylation of Ser827 on Par-3 was increased with elevation of aPKC activity. It was reported that aPKC phosphorylates Ser827 on Par-3, which reduces the stable interaction of Par-3—aPKC.\(^1,4\)

aPKC is activated by direct binding of PtdIns(3,4,5) to its cysteine-rich domain.\(^2,6\) PI3-kinase signaling is characterized by the stimulated production of the second messenger, PtdIns(3,4,5)\(_3\). PtdIns(3,4,5)\(_3\) activates PDK1, which
potentially activates aPKC and Akt.\textsuperscript{25,26} We revealed that the phosphorylation levels of aPKC and Akt were significantly increased by CCl4 treatment (Figure 5). These data indicated that aberrant activation of aPKC in CCl4-treated liver was induced by PI3-kinase signaling.

The activity of PTEN, the PtdIns(3,4,5)P3 phosphatase, is inhibited by oxidative stress, which causes an increase in cellular PtdIns(3,4,5)P3 levels.\textsuperscript{27} This suggests that oxidative stress is able to activate PI3-kinase—dependent signaling. Thus, CCl4-mediated activation of PI3-kinase signaling might activate aPKC.

Ultrastructural analysis revealed that the bile canalicular lumen was extended in CCl4-treated rat livers (Figure 2). In freeze-fracture replicas, analysis revealed that extrahepatic cholestasis is observed in the dilatation of the bile canalicular space.\textsuperscript{4} aPKC kinase activation induced expansion of the apical lumen in cultured epithelial cells through enhanced de novo apical exocytosis.\textsuperscript{3,5} We previously found that Par-3 knockdown results in a delay of apical protein carrier vesicle transport and leads eventually to delocalization of the apical membrane.\textsuperscript{8} Furthermore, interaction between Par-3 and aPKC is required for targeting of these protein carrier vesicles to primordial junction structures and normal apical domain development, such as organized lumen formation.\textsuperscript{8} Par-3 plays a critical role in regulating centrosome orientation for bile canalicus formation, but not its maintenance, in cultured rat hepatocytes.\textsuperscript{11} In this study, we found that Par-3—aPKC may play a crucial role in the maintenance of the bile canalicus lumen. This finding would provide novel insights into the understanding of maintenance and disturbance of the bile canalicus lumen. In CCl4-treated hepatocytes, the apical protein P-glycoprotein was localized to the bile canalicus (Supplemental Figure S3A). In addition, ultrastructural analysis revealed that apical microvilli formation was not altered (Figures 2 and 3). These data suggested that the apical domain was not affected. However, knockdown of Par-3 in epithelial cells revealed significant impairment of apical domain development.\textsuperscript{8}

CCl4 treatment induces accumulation of lipid-like materials and secondary lysosomes in hepatocytes.\textsuperscript{18,39} Par-3 and aPKC bind directly to phosphatidylinositol lipids.\textsuperscript{26,43,44} Par-3 and aPKC may interact with CCl4-induced lipid droplets within hepatocytes and translocate from TJs to the cytoplasm. Thus, it is possible that Par-3 and aPKC are attracted to the accumulated lipids in the cytoplasm (Figure 7). We found that CCl4-mediated bile canalicular lumen expansion was caused by aberrant activation of aPKC. Administration of vitamin E effectively prohibited CCl4-induced cytoplasmic localization of Par-3 and aPKC in rat liver. In fact, vitamin E blocked CCl4-induced increase in 4-hydroxy-2-nonenal adducts and preserved liver morphology (Figure 6). Our findings identified a novel effect of vitamin E in oxidative stress—mediated cell injury, but further analysis is needed.

Our results suggested that CCl4-mediated oxidative stress disturbed Par-3—aPKC interaction and led to a defect in hepatocyte polarity by aberrant activation of aPKC, indicating a link between the disturbance of cell polarity and cholestasis progression by oxidative stress. Further studies will clarify the molecular mechanisms underlying primary biliary cirrhosis, primary sclerosing cholangitis, and progressive intrahepatic cholestasis.

Acknowledgment

We thank Johbu Itoh for technical assistance with confocal laser microscopy.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.12.015.

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