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Inhibition of hepatic mitochondrial aldehyde dehydrogenase by carbon tetrachloride through JNK-mediated phosphorylation*

Kwan-Hoon Moon¹, Young-Mi Lee^{1,2}, and Byoung-Joon Song^{1,**}

¹ Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD, USA

² Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University, Iksan, Korea

Abstract

The aim of this study was to investigate the inhibitory mechanism of mitochondrial aldehyde dehydrogenase (ALDH2) by carbon tetrachloride (CCl₄). CCl₄ administration caused marked hepatocyte ballooning and necrosis in the pericentral region. CCl₄ also inhibited hepatic ALDH2 activity in a time-dependent manner without altering the protein level, suggesting ALDH2 inhibition through covalent modifications such as phosphorylation by JNK. To demonstrate phosphorylation, isoelectric points (pI) of ALDH2 in CCl₄-exposed rats were compared to those of untreated controls. Immunoblot analysis revealed that immunoreactive ALDH2 bands in CCl₄-exposed rats were shifted to acidic pI ranges on two-dimensional electrophoresis (2-DE) gels. Incubation with alkaline phosphatase significantly restored the suppressed ALDH2 activity with concurrent alkaline pI shifts of ALDH2 spots. Both JNK and activated JNK were translocated to mitochondria following CCl₄ exposure. In addition, incubation with catalytically active JNK led to significant inhibition of ALDH2 activity with acidic pI shifts on 2-DE gels. Furthermore, immunoprecipitation followed by immunoblot analysis with anti-phospho-Ser-Pro antibody revealed phosphorylation of Ser residue (s) of ALDH2. These results collectively indicate a novel underlying mechanism that CCl₄ exposure activates JNK, which translocates to mitochondria and phosphorylates ALDH2, contributing to inhibition of ALDH2 activity accompanied with decreased cellular defense capacity and increased lipid peroxidation.

Keywords

Mitochondrial aldehyde dehydrogenase; phosphorylation; JNK; CCl₄; lipid peroxidation; acute liver damage; rat liver

Introduction

Mitochondrial class 2 aldehyde dehydrogenase (ALDH2) is a key enzyme in cellular protection against oxidative damage. It efficiently metabolizes acetaldehyde produced during ethanol metabolism [1], as well as toxic lipid peroxides such as 4-hydroxynonenal (HNE) and

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**To whom correspondence should be addressed: Dr. B. J. Song, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, Maryland, 20892-9410, Phone: +1-301-496-3985; Fax: +1-301-594-3113; bjs@mail.nih.gov.

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malondialdehyde (MDA), which are produced after exposure to toxic chemicals [2,3]. Furthermore, ALDH2 exhibits an esterase activity for nitroglycerin analogues, which are used to treat congenital cardiovascular diseases [4,5]. Because of the protective role of ALDH2 in the metabolism of toxic aldehydes, inactivation of ALDH2 by various chemicals such as acetaminophen and carbon tetrachloride (CCl₄) [6–9] or during various disease states [10–12] is likely to cause marked accumulation of toxic aldehydes, leading to increased susceptibility toward tissue damage. It is also known that the *ALDH2* gene is highly polymorphic, especially in East Asian populations [13,14]. In fact, people with a dominant negative mutation in the *ALDH2* gene have a higher risk for oral/esophageal cancer [15,16], hepatic cancer [17,18], and myocardial infarct [19,20].

CCl₄ is a well-established hepatotoxin which is widely used to study the mechanism of toxic liver injury such as acute necrosis and chronic fibrosis in laboratory animals [21,22]. It is also known to be metabolized by the ethanol-inducible CYP2E1 [23]. Following its metabolism, free radical metabolites attack cellular membranes, producing lipid peroxidation which leads to severe necrosis in the pericentral regions of the liver [21,22]. Hjelle et al. [6,7] previously reported that ALDH2 activity is inhibited by CCl₄ exposure, although the detailed mechanism of its inhibition is still unknown.

Mitogen-activated protein kinases (MAPK) play important roles in regulating the rate of cell growth (or proliferation) and cell death, depending on the cellular contexts. One of the MAPKs is c-Jun N-terminal protein kinase (JNK), which is involved in liver injury caused by CCl₄ [24] and acetaminophen [25,26]. JNK-mediated cell damage has been also observed after exposure to ionizing radiation [27,28] and many other potentially toxic substances [29–31] as well as under various pathological conditions [32,33]. The selective activation of the JNK pathway after CCl₄ exposure induces apoptosis/necrosis of hepatocytes in the liver [34]. Although JNK is activated while ALDH2 is inhibited after exposure to CCl₄, the direct relationship between activated JNK and ALDH2 has not been examined. Therefore, we hypothesized that mitochondrial ALDH2 is inhibited through JNK-mediated phosphorylation of Ser or Thr residues following CCl₄ exposure. The aim of this study was to investigate whether CCl₄ inhibits ALDH2 activity through JNK-mediated phosphorylation.

Materials and methods

Chemicals and other materials

NAD⁺, propionyl aldehyde, pyrazole, and calf intestine alkaline phosphatase were purchased from Sigma (St. Louis, MO, USA). Specific antibodies to c-Jun, phospho-c-Jun (Ser63), JNK, phospho-JNK, phospho-Ser-Pro, or phospho-Thr-Pro were purchased from Cell Signaling Technology, Inc (Beverly, MA, USA). Specific monoclonal antibody to mitochondrial ALDH2 was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Recombinant JNK (catalog number 420105) was purchased from Calbiochem (San Diego, CA, USA).

Animal treatment

Young adult male outbred Sprague-Dawley rats (150–170 g) were obtained from Charles River Breeding Company (Wilmington, MA, USA) and kept in a 12 hour light-dark cycle with food and water *ad libitum* in accordance with NIH guidelines. After a single intraperitoneal injection of CCl₄ (5 per group) (2 g/kg body weight diluted in corn oil, 1:1) as described [35], the animals were euthanized at the indicated times.

Histological analysis of liver samples

A portion from the largest lobe of each liver, obtained from CCl₄-exposed rats collected at different time points, was fixed in 10% buffered formalin. After paraffin embedding and the

cutting of 5 μm slices, all sections were stained with hematoxylin and eosin (H&E). Histological evaluation was performed in a blinded manner.

Two-dimensional gel electrophoresis and immunoblot analysis

Mitochondrial fractions were prepared from pooled rat livers ($n=5/\text{group}$) from the different treatment groups using differential centrifugation followed by 3 separate washing steps, as previously described [36,37]. Mitochondrial proteins were dissolved in 2-DE gel buffer before isoelectric focusing analysis was performed on dry immobilized pH gradient strips (nonlinear gradient of pH 3–10) as described [36] and subjected to immunoblot analysis with specific anti-ALDH2 antibody and enhanced chemiluminescence detection.

Determination of the levels of lipid peroxidation, rate of hydrogen peroxide production, ALDH2 activity, and JNK activity

Malondialdehyde (MDA) levels were measured using a Lipid Peroxidation Assay kit (Calbiochem) per manufacturer's instructions. The level of hydrogen peroxide produced in isolated mitochondria was determined by using the Amplex® Red hydrogen peroxide assay kit obtained from Molecular Probes (Eugene, OR, USA) by following the manufacturer's instructions. The rate of hydrogen peroxide production was calculated by measuring the increased fluorescence value during 30 min incubation with 0.5 mg mitochondrial protein and expressed as $\mu\text{M H}_2\text{O}_2$ produced/hr/mg-protein [38]. ALDH2 activity was measured by quantifying the production of NADH at 340 nm as previously described [39,40]. JNK activity in the cytosolic fractions was measured using the nonradioactive JNK assay kit (Cell Signaling) following the manufacturer's instruction. A phospho-c-Jun (Ser63) antibody was used to measure JNK activity by immunoblot analysis after immunoprecipitation with anti-c-Jun antibody conjugated agarose beads.

Intensity data processing and statistical analysis

The intensity of immuno-recognized ALDH2 bands on 2-DE gels was determined by using a gel digitizing software (UN-SCAN-IT™, Orem, Utah, USA), as previously described [37]. All data in this report represent the results from at least three separate experiments, unless stated otherwise. Statistical analyses were performed using the Student's t test and $p<0.05$ was considered statistically significant. Other methods not described were the same as reported previously [29–31,36,37].

Results

Hepatic damage and oxidative stress following CCl_4 exposure

To assess hepatocellular damage in CCl_4 -exposed rat liver, liver histopathology analysis was performed. H & E staining data showed that normal liver histology was observed in untreated control and rats exposed to CCl_4 for 2 h and 4 h. However, marked hepatocyte ballooning and necrosis in the centrilobular region was observed in 16 h- CCl_4 exposed rat liver (Fig. 1A). Consistent with the histological data, the level of cytochrome c detected in the cytoplasm [31] was markedly elevated at 16 h after CCl_4 exposure compared to that of control (data not shown). Our results are in agreement with previous reports [21,22,24]. To demonstrate elevated oxidative stress in CCl_4 -exposed rat liver, the rate of mitochondrial hydrogen peroxide production were measured. The rate of hydrogen peroxide production was significantly increased after CCl_4 exposure at all time-points (Fig. 1B).

Inactivation and phosphorylation of ALDH2 after CCl_4 exposure

Chronic or acute CCl_4 treatment markedly elevates the rate of lipid peroxidation, which contributes to centrilobular necrosis in the liver of many species and eventually lead to fibrosis/

cirrhosis [21,22]. Specifically, mitochondrial ALDH2, with a low K_m for acetaldehyde and toxic lipid aldehydes [3,41,42], was shown to be inactivated by CCl_4 , however its inhibitory mechanism is poorly understood. To investigate the mechanism of CCl_4 -mediated ALDH2 inhibition, the hepatic levels of ALDH2 activity and its protein content were determined in rats exposed to a single dose of CCl_4 . As shown in Figure 2A, CCl_4 treatment significantly inhibited ALDH2 activity in a time-dependent manner. For example, ALDH2 activity was inhibited by 48% and 70%, respectively, at 2-h and 16-h after CCl_4 exposure. ALDH2 activity was not recovered even after incubation with 15 mM DTT (Fig. 2A) or Asc (data not shown), which effectively reduces oxidatively-modified cysteine residues such as sulfenic acid, disulfide, glutathionylated cysteines and S-nitrosylated cysteines [36,37]. These data indicate that oxidation of cysteine residue(s) does not appear to be critical in CCl_4 -mediated ALDH2 inactivation, although we can not rule out the possibility of hyperoxidation of cysteines to sulfinic and sulfonic acids. In addition, CCl_4 administration did not alter ALDH2 protein levels (Fig. 2B), suggesting that CCl_4 -mediated ALDH2 inhibition is likely due to another type of post-translational modification of ALDH2. Since ALDH2 is known to metabolize toxic lipid aldehydes, we also measured the levels of MDA, as an indicator of the level of lipid peroxidation, in CCl_4 -exposed tissues. As shown in Fig. 2C, the level of MDA was slightly increased in CCl_4 -exposed rats at 2-h post-treatment. However, the amounts of MDA were significantly increased after 4 and 16 h exposures, and these time points correlated with the time of suppressed ALDH2 activity.

Based on the activation of JNK by CCl_4 [34], we examined the potential role of JNK in phosphorylating ALDH2 in CCl_4 -exposed rats. To determine phosphorylation of ALDH2, we separated mitochondrial proteins on 2-DE gels and then subjected them to immunoblot analysis with an anti-ALDH2 antibody, as recently reported [31,43]. The immunoblot analysis (Fig. 2D) revealed that the ALDH2 immunoreactive bands in the CCl_4 -exposed livers were shifted to acidic pI ranges compared to those of control livers. At least 3 additional ALDH2 spots with acidic pIs were observed in CCl_4 -exposed liver samples compared to untreated liver samples, suggesting that ALDH2 might be covalently modified (i.e., phosphorylated). Our results suggest that ALDH2 spots in the acidic pI regions on 2-DE gels likely represent phosphorylated and inactive ALDH2 protein in CCl_4 -exposed rats. These results are similar to the acidic shift of the ALDH2 spots with markedly decreased ALDH2 activities in patients with hepatocellular carcinoma compared to those of normal control [17].

Dephosphorylation and reactivation of ALDH2 by alkaline phosphatase treatment

To directly demonstrate the role of phosphorylation in CCl_4 -mediated inhibition of ALDH2, we studied the effect of alkaline phosphatase on the suppression of ALDH2 activity subsequent to CCl_4 exposure (Fig. 3). ALDH2 activity was inhibited by 58.5% and 88.2% at 4-h and 16-h, respectively, after CCl_4 -exposure in the absence of alkaline phosphatase. However, after incubation with alkaline phosphatase, the suppressed ALDH2 activity was restored to 86.7% and 83.0% of untreated control samples, whereas the ALDH2 activity in control sample was not changed by addition of alkaline phosphatase. Furthermore, some ALDH2 spots with acidic pIs disappeared or shifted back to alkaline pI ranges on 2-DE gels in the samples exposed to CCl_4 for 4-h and 16-h (Fig. 3B and C, respectively). The quantitative densitometric analysis of each immunoreactive ALDH2 spot (e.g., spots 3 and 4 in Figs. 3B and 3C) confirmed that the ALDH2 spots significantly shifted back to alkaline ranges after incubation with alkaline phosphatase (Fig. 3D). These results indicate that ALDH2 activity is inhibited by phosphorylation following CCl_4 treatment and that the activity can be restored by dephosphorylation.

Translocation of activated JNK to the mitochondria and phosphorylation of ALDH2 after CCl₄ exposure

To determine the potential role of mitogen activated protein (MAP) kinase in phosphorylating ALDH2 after CCl₄-exposure, we determined the activity of JNK, p38 kinase, or ERK in CCl₄-exposed tissues by using the specific antibody to phospho-c-Jun, phospho-p38 kinase, or phospho-ERK. Consistent with the previous result [34], p38 kinase and ERK were not activated in CCl₄-exposed samples (data not shown). However, JNK activity, as indirectly measured by the phospho-c-Jun level (Figure 4A, right panel), was elevated after CCl₄ treatment in a time-dependent manner without any discernable changes in the levels of c-Jun (left panel) (Fig. 4A). To further address the question of whether mitochondrial ALDH2 is regulated through JNK-mediated phosphorylation, we also studied whether CCl₄ treatment induces JNK translocation to the mitochondria. Washed mitochondrial fractions from CCl₄-exposed rat livers were subjected to immunoblot analysis with a specific anti-JNK or anti-phospho-JNK antibody. Figure 4B shows that increased amounts of JNK (top panel) and phospho-JNK (middle panel) are associated with the mitochondria, indicating that both activated (phosphorylated) JNK1 and JNK2 proteins were translocated to the mitochondria following CCl₄ exposure. The levels of α -ATP synthase are presented to demonstrate equal loading of protein samples (bottom panel).

JNK is a proline-directed Ser/Thr protein kinase, which selectively phosphorylates serine and/or threonine residue(s) that are followed by proline [31], and ALDH2 contains four potential sites (Ser-Pro or Thr-Pro) for JNK-mediated phosphorylation. Therefore, we performed immunoblot analysis with the anti-phospho-Ser-Pro or anti-phospho-Thr-Pro antibody to determine the phosphorylation site(s) after ALDH2 was immunoprecipitated from each sample indicated. The immunoblot results showed that one ALDH2 band was detected using the anti-phospho-Ser-Pro antibody (Fig. 4C, right panel) with equal levels of ALDH2 in all of the samples (left panel). However, no band was detected with the anti-phospho-Thr-Pro antibody (data not shown), although this antibody clearly recognized phosphorylated Thr residues of Bax [31]. Taken together, these results indicate that ALDH2 could be inhibited possibly through JNK-mediated phosphorylation of Ser residues in CCl₄-exposed tissues.

Inhibition and phosphorylation of ALDH2 by recombinant JNK

To directly demonstrate the role of JNK in phosphorylation and subsequent inhibition of ALDH2, liver mitochondrial proteins from untreated control rats were incubated with recombinant JNK for 60 min. The levels of ALDH2 activity and phosphorylation were then determined by activity measurement and 2-DE gel analysis, respectively. Although the recombinant JNK (Calbiochem) was not phosphorylated to become fully-active [31], it was still catalytically active and suitable for our experiments. Following the incubation of mitochondrial proteins with JNK, ALDH2 activity was decreased by 36.0% (Fig. 5A). The low level of ALDH2 inhibition could result from the relatively small amount of ATP (0.2 mM) used in the assay condition. Nonetheless, additional immunoreactive ALDH2 spots appeared or intensified in the acidic pI ranges on 2-DE gels (Fig. 5B), further supporting that JNK phosphorylates ALDH2. Quantitative densitometric analysis confirmed a significant shift of immunoreactive ALDH2 spots to acidic pI ranges (i.e., spots 3 and 4) upon JNK incubation (Fig. 5C). Consistent with the results in Fig. 4C, immunoblot analysis with anti-phospho-Ser-Pro antibody recognized the immunoprecipitated ALDH2, which was pretreated with recombinant JNK. However, this anti-phospho-Ser-Pro antibody did not detect control ALDH2 protein (Fig. 5D, right panel), which was recognized by the anti-ALDH2 antibody (left panel). These results indicate that Ser residue(s) of ALDH2 in untreated control sample was phosphorylated after incubation with JNK. Collectively, these results strongly indicate that CCl₄ activated JNK, which is translocated to mitochondria, phosphorylates and thus inhibits ALDH2.

We also evaluated whether the activity of other ALDH isozymes such as cytosolic ALDH1 was inhibited by CCl₄ exposure. We hypothesized that the activity of cytosolic ALDH1 isozyme can also be inhibited by JNK-mediated phosphorylation in CCl₄-exposed rats because ALDH1 contains two Ser residues, that can be potentially phosphorylated by JNK. Contrary to our hypothesis, the activity of cytosolic ALDH1 was not significantly inhibited in CCl₄-treated rats exposed for 2, 4, or 16 h compared to untreated control (Fig. 6A). In addition, the cytosolic ALDH1 activity was not inhibited even after the cytosolic proteins were pre-incubated with recombinant JNK protein (Fig. 6B). In contrast, the mitochondrial ALDH2 activity was inhibited under the same condition (data not shown), as similar to the data shown in Fig 5A. Furthermore, we did not detect any additional ALDH1 spots in the acidic pI range on 2-DE gels even after incubation with JNK followed by immunoblot analysis of the immunoprecipitated ALDH1 protein (not shown). These results suggest that ALDH1, which contains two Ser residues compared to three Ser sites (in ALDH2) that are potentially phosphorylated by JNK (Fig. 6C), may not be modified by JNK after CCl₄ exposure. As summarized in Fig. 7, combinations of CCl₄-mediated activation of cell-death promoting JNK, calcium mobilization, mitochondrial dysfunction, and elevated levels of lipid peroxides through decreased ALDH2 activity are likely to contribute to acute liver damage [21,22,24].

Discussion

Mitochondrial ALDH2 is involved in the metabolism of carbonyl compounds with a very low K_m for acetaldehyde and other lipid aldehydes which are highly reactive and toxic. ALDH2 is known to be inhibited by many chemicals such as disulfiram, HNE, MDA, acetaminophen, ecstasy (3,4-methylenedioxymethamphetamine), and CCl₄ [6,7,9,37,44,45]. Its activity is also decreased in many pathological conditions such as alcoholic fatty liver, hepatic cancer, aging, and hepatic ischemia-reperfusion injury [16–18,36,40,46]. Under these conditions, inhibition of ALDH2 is expected to lead to accumulation of cytotoxic MDA and HNE, which can cause cell death through activation of JNK and/or p38 kinase [29]. However, the detailed mechanisms of ALDH2 inhibition by these chemicals including CCl₄ or under pathological conditions such as hepatocellular cancer patients are poorly understood.

The aim of this study was to elucidate the mechanism by which ALDH2 is inhibited after exposure to CCl₄. Although CCl₄ treatment caused rapid degradation of CYP2E1 [35], the amount of ALDH2 protein was unaltered by CCl₄ exposure. These results suggest that inhibition of ALDH2 activity can result from covalent modification(s) of key amino acid residues of ALDH2 such as oxidation, phosphorylation and adduct formation with lipid peroxides. In general, oxidative modifications of Cys residues of ALDH2 can theoretically undergo the following modifications: oxidations to sulfenic acid, sulfinic acid and sulfonic acid, disulfide, GSH-dependent *S*-glutathionylation, NO-dependent *S*-nitrosylation, and NO-independent ADP-ribosylation, as previously discussed [36,37,39,40]. Incubation of reducing agents such as DTT and ascorbate (Asc) completely restored the suppressed ALDH2 activity in alcohol-fed rats [36,46] and ecstasy-exposed rats [37], suggesting a reversible oxidative modification of Cys residue(s) in these models. However, addition of DTT or Asc did not restore the suppressed ALDH2 activity in CCl₄-exposed rats (Fig. 2A), suggesting a limited role of oxidative modifications of Cys residue(s). These results indicate another type of post-translational modification. In fact, Cys residues in ALDH2 could also be covalently modified by the reactive MDA and HNE, as demonstrated with HNE [45], since the levels of these lipid aldehydes are significantly elevated after CCl₄ exposure [2]. Although we can not rule out ALDH2 inhibition through the formation of adducts with MDA and/or HNE or hyperoxidation of Cys to sulfinic/sulfonic acids which can not be easily reversed by DTT, our data also show another mechanism of ALDH2 modulation via phosphorylation by JNK activated after CCl₄ exposure [34]. It is possible that other protein kinases activated after CCl₄ exposure can phosphorylate ALDH2 at different amino acid residues, resulting in inactivation of ALDH2,

although we did not perform this task. In contrast, Thr¹⁸⁵, Thr⁴¹² and Ser²⁷⁹ of ALDH2 have been shown to be phosphorylated by protein kinase Ce, resulting in activation of ALDH2 and protection against ischemic damage to the heart [47]. To demonstrate the phosphorylation and inactivation of ALDH2 following CCl₄ exposure, we used 2-DE gel analysis followed by immunoblot analysis by monitoring pI shifts of ALDH2 in different mitochondrial samples. Our current results indicate that ALDH2 can be inhibited through phosphorylation following CCl₄ exposure based on the following evidence: 1) marked inhibition of ALDH2 activity concurrent with acidic pI shifts in CCl₄-exposed rats compared to untreated control (Fig. 2); 2) significant inhibition of ALDH2 activity after incubation with recombinant JNK as well as acidic pI shifts (Fig. 5); 3) restoration of ALDH2 activity with alkaline pI shifts after incubation with alkaline phosphatase (Fig. 3); and 4) immuno-recognition of phosphorylated Ser residue (s) of ALDH2 with a specific anti-phospho-Ser-Pro antibody (Fig. 4).

Our current results obtained with CCl₄-exposed rat liver samples are similar to the results obtained from hepatocellular carcinomas (HCC) patients in which these individuals had markedly reduced ALDH2 activity with additional ALDH2 spots in the acidic pI ranges [17, 18]. Since other toxic agents such as acetaminophen [25,26,48], daunomycin [11], and HNE [29] or UV irradiation [28,31] can also activate JNK and/or p38 kinase, we expect that ALDH2 activity could also be inhibited through phosphorylation in a manner similar to the mode of CCl₄-mediated ALDH2 inhibition. In addition, we predicted that the activity of cytosolic ALDH1 isozyme could be inhibited by a similar inhibitory mechanism through JNK-mediated phosphorylation after CCl₄ exposure because two out of the three potential phosphorylation sites (Ser-Pro residues) of ALDH2 are also conserved in ALDH1. However, contrary to our prediction, the cytosolic ALDH1 activity was not inhibited in CCl₄-exposed tissues, suggesting specific inhibition of ALDH2 by CCl₄.

Our data also showed that both JNK and activated JNK are translocated to the mitochondria, likely promoting phosphorylation of ALDH2 (and other mitochondrial proteins), leading to ALDH2 inhibition following CCl₄ exposure. Structural analysis of the primary structure of rat ALDH2 shows that ALDH2 contains at least one putative JNK binding domain of R/K₂₋₃-X₁₋₆-L/I-X-L/I [49] at Lys²⁶³-Arg²⁶⁴-Val-Thr-Leu²⁶⁷-Glu-Leu²⁶⁹. These results suggest that ALDH2 can interact with JNK and can thus become its substrate (target) protein. Binding of JNK to this JNK binding domain of ALDH2 could theoretically interfere with the critical interaction between each subunit (Arg²⁶⁴ and Glu⁴⁸⁷) within the homotetrameric ALDH2 [50], likely leading to inhibition of ALDH2 activity. In addition, analysis of phosphorylation sites by the NetPhos software (version 2.0 available at <http://www.cbs.dtu.dk/services/NetPhosK>) predicts that ALDH2 contains at least 4 potential phosphorylation sites (i.e., Ser⁷⁴-Pro, Thr¹⁹⁷-Pro, Ser²⁷³-Pro, and Ser⁴⁶³-Pro) of JNK, which phosphorylates Ser/Thr residues followed by Pro residues. Based on the selective immunoreactivity to the anti-phospho-Ser-Pro antibody and not with the anti-phospho-Thr-Pro antibody, which recognizes phosphorylated Thr residue [31] of Bax (data not shown), we believe that at least 3 Ser residues (Ser⁷⁴, Ser²⁷³, and Ser⁴⁶³) can be phosphorylated by activated JNK. It is also expected that other MAP kinases activated by other hepatotoxic agents can phosphorylate ALDH2 (and other ALDH isozymes based on conserved amino acid sequences) via similar mechanism as demonstrated in this study. Phosphorylation of these Ser residues may cause conformational change, contributing to the inhibition of ALDH2 activity via alterations in substrate or cofactor binding. In fact, this prediction is consistent with a report by Rout et al [51] where removal of Ser⁷⁴, one of the 3 potential Ser residues of JNK-mediated phosphorylation as listed above, in rat liver ALDH2 affects the coenzyme binding, leading to ALDH2 inhibition. However, based on the differential activity data of ALDH isozymes (Figs. 2, 3 and 5 versus Fig. 6) and the structural comparison of the potential sites of JNK-mediated phosphorylation in rat ALDH2 and ALDH1 proteins (Fig. 6C), it is likely that Ser⁴⁶³-Pro of ALDH2 is a major site of JNK-mediated phosphorylation, although this prediction needs to be

confirmed by experimental data. Consequently, the levels of toxic lipid peroxides (such as MDA and HNE) are elevated contributing to acute damage (necrosis) of hepatocytes, as summarized in Figure 7. In addition, elevated MDA and HNE may activate liver stellate cells and thus increase the production of collagen, leading to fibrosis.

Although the critical role of JNK in apoptotic cell death or survival pathway signaling has been actively investigated, its role in phosphorylation of mitochondrial proteins and alteration of mitochondrial physiology including energy metabolism and apoptosis has not been extensively studied. However, recent reports have clearly demonstrated that activated JNK actually translocates to the mitochondria to phosphorylate mitochondrial proteins, altering their functions. For instance, Kufe and coworkers reported that activated JNK was translocated to mitochondria to interact with Bcl-x_L and phosphorylate its 2 Thr residues, leading to apoptosis in cultured cells exposed to ionizing radiation [27]. In addition, Hanawa et al. [48] reported that activated JNK was translocated to mitochondria to induce mitochondrial permeability transition and inhibit mitochondrial respiration, contributing to acute hepatocyte injury in acetaminophen-exposed mice. Although the mitochondrial target proteins phosphorylated by JNK has not been identified in this study, another study suggests that JNK phosphorylates several mitochondrial proteins when mitochondrial proteins were incubated with active JNK [43]. The JNK-catalyzed target proteins, identified by the shift of their pI values on 2-DE gels followed by N-terminal peptide sequencing, include ATP synthase α and β -subunits (inner membrane proteins), pyruvate dehydrogenase E1 β subunit and Hsp60 (both matrix proteins). These data suggest that many other mitochondrial proteins can become target proteins of JNK, as long as they contain the putative JNK binding domain [49]. Our current results show that ALDH2, a mitochondrial matrix protein, is a target protein of JNK. Furthermore, phosphorylation of key mitochondrial proteins by JNK (and/or other MAP kinases) could alter functions of the target proteins, leading to increased lipid peroxidation, mitochondrial permeability change with decreased bioenergetics [48], cytochrome c release [43], and reduced anti-apoptotic ability [27], all of which contribute to acute cell death (apoptosis/necrosis). It would be of interest to find out what other mitochondrial proteins could become the target proteins of translocated JNK (and other MAP kinases) under conditions of cellular damage. Systematic investigation of other mitochondrial proteins, which can be phosphorylated by activated JNK, and elucidating the functional consequences of the phosphorylations could also provide important clues to determine the mechanism of cell/tissue damage induced by CCl₄ and other toxic chemicals or under pathological conditions such as ischemia-reperfusion.

In conclusion, our results demonstrate for the first time that mitochondrial ALDH2 in rat liver was inhibited through JNK-mediated phosphorylation following CCl₄ exposure, leading to increased oxidative stress and lipid peroxides, ultimately contributing to acute hepatic damage.

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Abbreviations

ALDH2	mitochondrial class 2 aldehyde dehydrogenase
Asc	sodium ascorbate
CCl ₄	carbon tetrachloride

DTT	dithiothreitol
JNK	c-Jun <i>N</i> -terminal protein kinase
HNE	4-hydroxy-2-nonenal
MDA	malondialdehyde
ROS/RNS	reactive oxygen/nitrogen species
2-DE	two-dimensional electrophoresis
MAPK	mitogen- activated protein kinase
pI	protein isoelectric point

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Fig. 1.

Increased hepatic damage and oxidative stress following CCl₄ administration. (A) Typical hematoxylin and eosin (H&E) stained liver slides are presented for the untreated control and the CCl₄-exposed group for 16 h (magnification 100 ×) (B) Rate of mitochondrial hydrogen peroxide production was determined in untreated control and rats exposed to CCl₄ for 2, 4, and 16 h. * and **, significantly different (**p*<0.05 and ***p*<0.005) from the control.

Fig. 2.

Inhibition and phosphorylation of ALDH2 by CCl₄ exposure. (A) Mitochondrial proteins (0.5 mg/assay) were incubated with or without 15 mM DTT for 30 min before ALDH2 activity was determined for the samples indicated. One unit of ALDH2 activity represents a reduction of 1 μmol NAD⁺/min/mg protein. * and **, significantly different (**p*<0.005 and ***p*<0.0005) from the control. (B) Equal amounts of mitochondrial proteins (20 μg/well) from the indicated samples were subjected to SDS-PAGE and immunoblot analysis with the anti-ALDH2 antibody. (C) Hepatic MDA levels from the same set of samples were measured. * and **, significantly different (**p*<0.05 and ***p*<0.001) from the control. (D) Mitochondrial proteins (0.1 mg/analysis) from control and rats exposed to CCl₄ for 16 h were separated on 2-DE gels

and then subjected to immunoblot analysis with anti-ALDH2 antibody. pI ranges on 2-DE gels are shown below, while the major immunoreactive ALDH2 protein spots are indicated above.

Fig. 3.

Dephosphorylation and re-activation of ALDH2 after incubation with alkaline phosphatase. (A) Mitochondrial proteins (0.5 mg/assay) were incubated with or without alkaline phosphatase (40 units) for 1 h, pH 9.8 at 37 °C before ALDH2 activity was measured for the indicated samples. One unit of alkaline phosphatase represents 1 μ mol of 4-nitrophenyl phosphate/min at pH 9.8 and 37 °C. *significantly different ($p < 0.001$) from the control; **significantly different ($p < 0.005$) from the corresponding CCl₄-exposed samples. (B, C) Mitochondrial proteins (0.1 mg/analysis) from the same samples shown in A were separated on 2-DE gels and then subjected to immunoblot analysis with anti-ALDH2 antibody. (D) The relative ratio of immunoreactive ALDH2 spots was calculated by dividing the density of spot 3 or 4 with

the total spot density of each gel shown in B and C. **Significantly different ($p < 0.005$) from the corresponding spots without alkaline phosphatase treatment.

Fig. 4.

Translocation of JNK to the mitochondria and ALDH2 phosphorylation following CCl₄ exposure. (A) To measure JNK activity, cytosolic proteins (0.5 mg/assay) from indicated samples were initially immunoprecipitated with anti-c-Jun fusion protein beads with gentle head-to-tail rotation overnight at 4 °C. The immunoprecipitated c-Jun protein was incubated with the kinase reaction buffer (pH 7.5) containing 200 μ M ATP and 10 mM MgCl₂ for 30 min at 30 °C [25,29–31] before it was subjected to SDS-PAGE and immunoblot analysis with the specific anti-c-Jun (left) or anti-phospho-c-Jun antibody (right). (B) To demonstrate mitochondrial translocation of JNK, mitochondrial proteins (50 μ g/lane) from each indicated sample were subjected to SDS-PAGE and immunoblot analysis with the specific antibody to anti-JNK (top), phospho-JNK (middle), or α -ATP synthase (bottom). (C) Mitochondrial proteins (0.5 mg/sample) from each indicated sample were immunoprecipitated with the specific anti-ALDH2 antibody, separated on SDS-PAGE, and subjected to immunoblot analysis using the anti-ALDH2 antibody (left) or anti-phospho-Ser-Pro antibody (right).

Fig. 5.

Phosphorylation and inhibition of ALDH2 after incubation with catalytically active JNK. (A) Mitochondrial proteins from untreated control rats (0.5 mg/assay) were incubated with or without JNK (25 milli-units) in a buffer (pH 7.5) containing 200 μ M ATP and 10 mM MgCl_2 for 1 h at 30 $^{\circ}\text{C}$ before ALDH2 activity was measured. One unit of JNK represents 1.0 nmol of phosphorylation of recombinant c-Jun protein/min at 30 $^{\circ}\text{C}$, pH 7.5. *Significantly different ($p < 0.05$) from the control without JNK incubation. (B) Mitochondrial proteins (0.1 mg/analysis) from control rats were incubated with or without JNK (5 milli-units) for 1 h at 30 $^{\circ}\text{C}$ before they were separated on 2-DE gels and subjected to immunoblot analysis with the anti-ALDH2 antibody. (C) The relative ratio of immunoreactive ALDH2 spots was calculated by dividing the density of spot 3 or 4 with the total spot density of each gel shown in panel B. This figure represents a typical result from at least two different experiments. (D) Mitochondrial proteins from untreated control rats (0.5 mg/assay) were incubated with or without JNK (25 milli-units) in a buffer (pH 7.5) containing 200 μ M ATP and 10 mM MgCl_2 for 1 h at 30 $^{\circ}\text{C}$. ALDH2 proteins in untreated control or JNK-treated group were immunoprecipitated with the specific anti-ALDH2 antibody, separated on SDS-PAGE, and

subjected to immunoblot analysis using the anti-ALDH2 antibody (left) or anti-phospho-Ser-Pro antibody (right).

(A)

(B)

(C)

Protein	Peptide sequence			Swiss-Prot
ALDH1	QAFQIG ⁷⁵ <u>SP</u> WRTMDA	LELGK ²⁷⁴ <u>SP</u> CIVFAD	MILSAQ ⁴⁶⁴ CPFGGFKM	P51647
ALDH2	AAFQLG ⁷⁴ <u>SP</u> WRRMDA	LELGK ²⁷³ <u>SP</u> NIIMSD	DVFGAQ ⁴⁶³ <u>SP</u> FGGYKM	P11884

Fig. 6.

Little inhibition of cytosolic ALDH1 by CCl₄ exposure and structural comparison of the potential phosphorylation sites in the rat ALDH1 and ALDH2 proteins. (A) Cytosolic proteins (0.5 mg/assay) in indicated samples were used to determine the ALDH1 activity by the method recently described [52]. One unit of ALDH1 activity represents a reduction of 1 μ mol NAD⁺/min/mg protein. (B) Cytosolic proteins from untreated control rats (0.5 mg/assay) were incubated with or without JNK (25 milli-units) in a buffer (pH 7.5) containing 200 μ M ATP and 10 mM MgCl₂ for 1 h at 30 °C before ALDH1 activity was measured. (C) Structural comparison of the potential sites of JNK-mediated phosphorylation in the rat ALDH1 and

ALDH2 proteins. The canonical Ser-Pro sites in both ALDH1 and ALDH2 were marked with bold characters. Numerical numbers represent the amino acid positions.

Proposed mechanism of CCl₄-mediated acute cell death **Fig. 7.**

Proposed mechanism of CCl₄-mediated acute cell death. Metabolism of CCl₄ produces free radicals and reactive oxygen species, that activate (phosphorylate) JNK [31]. Activated JNK is then translocated to the mitochondria and phosphorylates mitochondrial target proteins including ALDH2 and Bcl-2. Inhibition of ALDH2 likely causes elevation of MDA and HNE, which can further activate JNK as a positive enforcer. Moreover, activated JNK can phosphorylate cytosolic Bax, which can be also translocated to the mitochondria to cause mitochondrial permeability change and cytochrome c release into the cytoplasm. Collectively, activated JNK, inhibition of ALDH2, elevated levels of lipid peroxides, and other events such as calcium mobilization [21,22] likely act in a concerted or synergistic manner to contribute to acute cell death.