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Post-translational modifications of mitochondrial aldehyde dehydrogenase and biomedical implications

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Abstract

Aldehyde dehydrogenases (ALDHs) represent large family members of $NAD(P)^+$-dependent dehydrogenases responsible for the irreversible metabolism of many endogenous and exogenous aldehydes to the corresponding acids. Among 19 ALDH isozymes, mitochondrial ALDH2 is a low K_m enzyme responsible for the metabolism of acetaldehyde and lipid peroxides such as malondialdehyde and 4-hydroxynonenal, both of which are highly reactive and toxic. Consequently, inhibition of ALDH2 would lead to elevated levels of acetaldehyde and other reactive lipid peroxides following ethanol intake and/or exposure to toxic chemicals. In addition, many East Asian people with a dominant negative mutation in *ALDH2* gene possess a decreased ALDH2 activity with increased risks for various types of cancer, myocardial infarct, alcoholic liver disease, and other pathological conditions. The aim of this review is to briefly describe the multiple post-translational modifications of mitochondrial ALDH2, as an example, after exposure to toxic chemicals or under different disease states and their pathophysiological roles in promoting alcohol/drug-mediated tissue damage. We also briefly mention exciting preclinical translational research opportunities to identify small molecule activators of ALDH2 and its isozymes as potentially therapeutic/preventive agents against various disease states where the expression or activity of ALDH enzymes is altered or inactivated.

Keywords

Aldehyde dehydrogenases; post-translational modifications; cellular defense; drug toxicity; disease states; translational research

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1. Introduction

Various reactive aldehydes can be produced from endogenous and exogenous precursors under many different pathophysiological states as well as following exposure to potentially toxic agents including abused substances such as alcohol (ethanol) and cocaine. For instance, in mammals, toxic acetaldehyde can be produced as an intermediate during alcohol metabolism catalyzed by alcohol dehydrogenase (ADH)1 before it is further oxidized to acetic acid by mitochondrial aldehyde dehydrogenase 2 isozyme (ALDH2). Both ADH and ALDH2-mediated reactions require NAD^+ as a cofactor. Upon exposure to exogenous toxic chemicals [e.g., carbon tetrachloride (CCl_4)] or under physiological conditions [e.g., UV exposure], lipid peroxidation takes place due to increased oxidative stress. As a result, the cellular levels of toxic lipid peroxides such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) are elevated. These aldehydes are highly reactive and thus interact with cellular macromolecules including DNA and proteins [1,2]. Consequently, normal functions of DNA and protein targets are negatively affected, resulting in DNA damage/deletion/mutation and inactivation of proteins, respectively. In severe cases, these toxic aldehydes can directly promote cell/tissue damage (via apoptosis and necrosis) through promoting a loss of mitochondrial potential and activating the mitochondria-dependent cell death pathways [2–5]. However, under normal conditions, these reactive aldehydes can be effectively managed by various antioxidants (e.g., glutathione and ascorbic acid) as well as different metabolic enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase (ALDH), aldo-keto reductase, aldehyde oxidase, glutathione transferase, etc [6–8].

Among the cellular protective enzymes listed above, ALDH isozymes (EC 1.2.1.3) represent $NAD(P)^+$-dependent enzymes involved in the metabolism (oxidation) of various toxic aldehydes of endogenous and exogenous origins into their corresponding acids. In general, the ALDH-mediated reactions are considered irreversible. The *ALDH* superfamily consists of at least 19 *ALDH* genes in the human genome and that mutations in some ALDH isozymes are associated with inborn errors with altered aldehyde metabolisms and thus increased susceptibility to certain disease states [9–11]. Although many ALDH isozymes share overlapping substrate specificities and tissue/subcellular distribution, they may have different kinetic parameters such as distinct K_m values and catalytic activities toward each substrate compound [9–11]. In addition, the biochemical properties of each homologous ALDH isozyme depend on the species examined. For instance, human cytosolic ALDH1A1 isozyme exhibits a high K_m value (a range of 170~190 μM) for acetaldehyde [12]. However, the K_m value for acetaldehyde in rodent ALDH1A1 is relatively low (a range of 12 ~ 17 μM measured at pH 7.5), and comparable to that of rodent ALDH2 (0.2 μM) [12]. Therefore, it is reasonable to consider that ALDH2 in humans and ALDH2/1A1 isozymes in rodents likely represent the major enzyme(s) responsible for the metabolism of acetaldehyde produced from ethanol metabolism [12–14].

Because of the important roles of ALDH isozymes in efficiently removing potentially toxic aldehyde compounds, their tissue/subcellular distribution, substrate specificity, regulation of gene expression, and biochemical properties of each ALDH isozyme have been extensively studied [see reviews 9–11]. In general, most ALDH isozymes are expressed in large amounts in the liver while extra-hepatic tissues usually contain lower amounts of ALDH

[1]The abbreviations used are: **AEN**, aminoethyl nitrate; **ALDH**, aldehyde dehydrogenase; **ALDH1A1**, cytosolic aldehyde dehydrogenase; **ALDH2**, mitochondrial low-Km aldehyde dehydrogenase 2; **CYP2E1**, ethanol-inducible cytochrome P450 2E1 isozyme; **DIGE**, fluorescence 2-D difference gel electrophoresis; **DTT**, dithiothreitol; **GSK-3**, glycogen synthase kinase-3; **GSNO**, nitrosoglutathione; **GTN**, glyceryl trinitrate; **4-HNE**, 4-hydroxynonenal; **I/R**, ischemia-reperfusion; **JNK**, c-Jun *N*-terminal protein kinase; **MDA**, malondialdehyde; **MDMA**, 3,4-methylenedioxymethamphetamine; **PI3K**, phosphatidyl-inositol-3-kinase; **PKCε**, protein kinase Cε isozyme; **PTM**, post-translational modification; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species; ***S*-NO-Cys**, *S*-nitrosylated Cys; **SNP**, single nucleotide polymorphism.

isozymes. However, certain tissues may contain a relatively large amount of a specific ALDH isozyme depending on the unique function of each tissue. For instance, ALDH5A1, NAD^+-dependent succinic semialdehyde dehydrogenase involved in the metabolism of a neurotransmitter GABA, is highly expressed in the brain compared to other non-neuronal tissues including the liver [15]. In contrast, ALDH7A1, responsible for cellular protection against salinity, dehydration, and hyper-osmotic stress and betaine aldehyde metabolism, is highly expressed in the kidney, heart, ovary, cochlea, and eye [9,16].

It is now known that the catalytic activities of ALDH2 and ALDH isozymes are usually suppressed under pathophysiological conditions through synergistic interaction between genetic factors (e.g., allelic variation/mutation/single nucleotide polymorphism (SNP)/copy number variation in *ALDH* genes) [14,17–23] and environmental factors (e.g., alcohol, drugs, smoking, high-fat diet, viral/bacterial infections, toxic chemicals, etc) [24–29] (Figure 1). However, the causal roles of ALDH isozymes in the disease states are incompletely understood. In addition, despite the well-established biochemical properties of each ALDH isozyme, it is poorly understood how these ALDH isozymes are suppressed under some pathological conditions or following exposure to potentially toxic chemicals. We hypothesized that the catalytic activities of ALDH isozymes could be suppressed through post-translational modifications (PTMs) because the amounts of ALDH protein contents seemed unchanged or altered in a small amount (compared with the marked inhibitions of the catalytic activity) after exposure to toxic chemicals including alcohol or following hepatic I/R injury [30–39]. In fact, the mechanisms of PTMs of ALDH isozymes and subsequent changes in their catalytic activities under various pathophysiological conditions have not been studied thoroughly. Therefore, the aim of this review is to briefly describe the multiple PTMs of ALDH isozymes, updating the previous report [40]. As an example, we describe several examples of PTMs of mitochondrial ALDH2 following exposure to various toxic chemicals or under different disease models/states and discuss their functional implications in promoting alcohol/drug-mediated mitochondrial dysfunction and tissue damage. Finally, we briefly mention translational research opportunities in identifying chemicals that can activate ALDH2 and isozymes and thus can be potentially used as preventive/supportive/therapeutic agents against various disease states where ALDH isozymes are inactivated.

2. Post-translational modifications of ALDH2 and ALDH isozymes

Under increased oxidative/nitrosative stress, many amino acids such as cysteine (Cys), methionine (Met), tyrosine (Tyr), proline (Pro), histidine (His), lysine (Lys), arginine (Arg), threonine (Thr), phenylalanine (Phe), and tryptophan (Trp) in most proteins can be covalently-modified and their proteins functions are altered (i.e., usually inhibited) [41–43]. It is also true that ALDH isozymes could be modified by various forms of PTMs under pathophysiological conditions, resulting in their inactivation. These PTMs include: oxidation, *S*-nitrosylation, phosphorylation, nitration, hyper-acetylation, glycosylation, adduct formation, etc. Consequently, the catalytic activities of the ALDH isozymes usually decreased following covalent modifications except for a few cases, as discussed later. We will briefly describe several types of PTMs observed in ALDH2, as an example, under different pathophysiological conditions.

A) Oxidative modifications of critical Cys residues of ALDH2 and isozymes

It is known that Cys residues in most proteins can undergo many different types of oxidative-modifications under oxidative/nitrosative stress [41–43]. The oxidative modifications include: oxidation to sulfenic acid [44], disulfide [45–47], sulfinic/sulfonic acids [48], NO- or peroxynitrite-dependent *S*-nitrosylation [49,50], NO-independent ADP-ribosylation [51], mixed disulfide formation with glutathione [52], cysteine [53], succinic

acid [54], etc. Site-directed mutagenesis of each Cys residue of ALDH2 followed by biochemical characterization demonstrated the active site Cys^{302} residue [55]. The structural alignment and comparison revealed that the active site Cys residue is 100% conserved among the 17 ALDH isozymes (see Table 1). Because of the suppressed ALDH2 and ALDH1 activities in alcoholic individuals [56,57] or rodents exposed to alcohol [30–35], which increases oxidative/nitrosative stress [58–61], it is expected that the critical Cys residues including the highly-conserved active site Cys could be oxidatively-modified and thus inactivated. In fact, our proteomics analyses showed that cytosolic ALDH1A1, mitochondrial ALDH2, and other isozymes such as ALDH5A1 and ALDH6A1 were oxidatively-modified in rats exposed to alcohol [32–35] or 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) [36] and after hepatic ischemia-reperfusion (I/R) injury [37]. The hepatic ALDH2 activities in these tissue samples were all inhibited but their activities were restored when the activity was measured after pre-incubation with a strong reducing agent dithiothreitol (DTT). To further characterize the nature of Cys modification, we performed immunoblot analysis of the immunoaffinity purified ALDH2 proteins by using the specific antibody against ALDH2 or *S*-NO-Cys. With the anti-ALDH2 antibody, we detected similar levels of ALDH2 protein (54 kDa) in all 3 mitochondrial samples [i.e., control, alcohol-exposed rats without DTT pre-treatment, and alcohol-exposed rats pretreated with DTT, respectively]. However, we did not detect any immunoreactive band in control rats with the anti-*S*-NO-Cys antibody. In contrast, one band (54 kDa - same as the immunopurified ALDH2 protein) was recognized in alcohol-exposed rats with the anti-*S*-NO-Cys antibody. However, the band recognized with the *S*-NO-Cys antibody disappeared when the mitochondrial proteins were pre-incubated with DTT. The immunoblot data positively correlated with the fluctuations of the ALDH2 catalytic activity in the absence or presence of DTT. All these data strongly suggest that mitochondrial ALDH2 was *S*-nitrosylated in alcohol-exposed rats [32]. Similar results were also observed with the ALDH2 protein in rat hepatoma H4IIE-C3 cells exposed to NO donors such as *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylpenicillamine, and 3-morpholinosydnonimine, respectively [31]. Incubation with DTT or another reducing agent glutathione-ethylester significantly restored the ALDH2 activity suppressed by each NO donor. Immunoblot analysis with anti-*S*-NO-Cys antibody showed one immunoreactive band (54 kDa, same size as ALDH2) in GSNO-exposed hepatoma cells. The *S*-nitrosylated ALDH2 band disappeared when GSNO-exposed cells were pretreated with glutathione-ethylester, confirming *S*-nitrosylation of ALDH2 [31]. Although we have not characterized the mechanism of ALDH2 inhibition in MDMA-exposed rats [36] or mice with hepatic I/R injury [37], it is likely that ALDH2 activities could be inhibited through *S*-nitrosylation of critical Cys residue(s) because the suppressed ALDH2 activities were recovered in the presence of DTT, as in the case of alcohol-exposed rats [32].

We observed that cytosolic ALDH1A1 and ALDH4A1 were oxidatively-modified in alcohol-exposed mice [33] when we performed a redox proteomics approach using biotin-*N*-maleimide as a sensitive probe for oxidatively-modified Cys residue(s) [30], as recently reviewed [62,63]. Therefore, we investigated whether cytosolic ALDH1 activity was also inhibited in alcohol-exposed rat liver, similar to the suppression of mitochondrial ALDH2. Our subsequent study revealed that cytosolic ALDH1 activity was inhibited in alcohol-exposed rat liver but restored by the presence of DTT, suggesting that cytosolic ALDH1 was reversibly inactivated [34]. Immunoblot analysis with the anti-*S*-NO-Cys antibody confirmed *S*-nitrosylation of ALDH1 protein based on the detection of an immunoreactive band (~55 kDa) in the alcohol-exposed rats and its disappearance after pre-incubation with DTT. Furthermore, the pattern of detection and disappearance of the immunoreactive *S*-NO-Cys band correlated with the ALDH1 activity changes in the absence and presence of DTT, respectively [34].

Recent data suggest that *S*-nitrosylation of Cys of certain proteins in cardiac tissues may play a protective role against oxidative tissue damage by preventing irreversible hyper-oxidation of Cys residues under increased oxidative stress [64,65], as observed in gram-positive bacteria including *Bacillus subtilis*, which lacks low molecular antioxidant glutathione [53]. Theoretically, the Cys residues of *S*-nitrosylated proteins including cardiac ALDH2 should be restored to free sulfhydryl groups when the levels of cellular antioxidants such as glutathione and ascorbate become elevated in the heart. Although this theory may be correct, however, it is unclear whether *S*-nitrosylated cardiac ALDH2 exhibits a decreased activity than the native protein. It is also unknown whether *S*-nitrosylated hepatic ALDH1A1 and ALDH2 observed in alcohol-exposed rodents represent a cellular protective mechanism (e.g., prevention from irreversible hyper-oxidation to sulfinic/sulfonic acids), as proposed for the *S*-nitrosylated proteins in cardiac tissues [64,65] or simply reflect the inactivated proteins *per se*. Since the liver possesses a high capacity of regenerating or refurbishing the antioxidants through activation of a protective transcription factor Nrf-2 and other genes, the latter case looks more physiological and consistent with the suppressed ALDH activities observed in human alcoholic individuals [56,57]. Thus, based on the conflicting views, the physiological role of *S*-nitrosylation of hepatic ALDH2 and other ALDH isozymes in alcoholic and nonalcoholic fatty liver or fibrotic/cirrhotic diseases need re-evaluation in the future. In this regard, it would be important to further characterize the covalent modifications of hepatic ALDH2 protein purified from alcoholic individuals compared to control subjects.

B) Phosphorylation of ALDH2 and isozymes

It is well-established that ethanol-inducible cytochrome P450 2E1 (CYP2E1) is a major enzyme responsible for the metabolism of various small molecule toxic compounds including ethanol, acetaminophen, CCl_4, dimethylnitrosamine, benzene, etc [58–61,66–68]. The acute toxicity by these agents was prevented or greatly alleviated in *Cyp2e1*-null mice [69] or pre-treatment with a CYP2E1 inhibitor [70,71]. Acute exposure of ethanol, acetaminophen, or CCl_4, increases oxidative/nitrosative stress while each compound markedly inhibits the activity of mitochondrial ALDH2 activity, contributing to mitochondrial dysfunction and severe liver injury (through apoptosis/necrosis) in the pericentral region [69,72]. Because of the ALDH inhibition, the levels of reactive lipid peroxides such as 4-HNE and MDA were increased. Consequently, these elevated toxic peroxides can overwhelm the cellular anti-oxidant defense system, interact with many proteins in different sub-cellular organelles including mitochondria, thus alter their functions, resulting in mitochondria dysfunction and cell death [1–5,42]. In addition, long-term chronic exposure of 4-HNE and MDA can activate hepatic stellate cells, which produce collagen and pro-fibrotic growth factors including transforming growth factor-β, leading to fibrosis and cirrhosis in the liver [73]. Furthermore, these reactive lipid aldehydes can interact with DNA [1,74], causing DNA damage, deletion, and mutations, ultimately contributing to carcinogenesis, as illustrated (Figure 2). Besides these scenarios, other events may also take place cell/tissue damage. For instance, increased oxidative/nitrosative stress can concurrently decrease the levels of anti-oxidants, inhibit anti-oxidant defensive enzymes including mitochondrial superoxide dismutase, activate c-Jun *N*-terminal protein kinase (JNK)-mediated cell death signaling pathway [5,75], stimulate STAT-1 mediated proinflammatory signaling pathway [76], etc. All these pathways may also contribute to mitochondrial dysfunction and cell/organ damage.

Despite these acute and chronic effects of various CYP2E1 substrate compounds, the inhibitory mechanism of ALDH2 by each compound is poorly understood. Acute administration of ethanol [75], acetaminophen [69,71], CCl_4 [77], 4-HNE [5], or troglitazone [78], a hepatotoxic non-CYP2E1 substrate compound, was shown to activate

JNK, contributing to cell death. Based on JNK activation by these compounds, we hypothesized that CCl_4 could inhibit ALDH2 through JNK-mediated phosphorylation. However, the direct relationship between the activated JNK and ALDH2 activity has not been studied. Therefore, we recently studied the direct role of JNK in phosphorylating and inactivating ALDH2 in CCl_4-exposed rat tissues [39].

Consistent with the earlier data [24,25], our results showed that CCl_4 inhibited hepatic ALDH2 activity in a time-dependent manner without altering its protein amount assessed by immunoblot analysis. In contrast, cytosolic ALDH1A1 was not inhibited by CCl_4 exposure. Moreover, addition of DTT did not restore the suppressed ALDH2 activity, suggesting that ALDH2 could be inhibited through irreversible covalent modifications such as phosphorylation, based on JNK activation by CCl_4 [77]. Since phosphorylated proteins are often detected in more acidic pI regions (i.e., acidic-shifts) than non-phosphorylated native proteins on 2-D gels (with little changes in their mobility on 1-D gels) [79], we performed 2-D gel analysis of ALDH2 protein in control and CCl_4-exposed rat liver mitochondria samples. Immunoblot analysis with the anti-ALDH2 antibody revealed that 3 ALDH2 spots were detected in corn oil-treated (vehicle) control samples. However, 2~3 additional immunoreactive ALDH2 spots in acidic pI regions were detected in CCl_4-exposed rats. Incubation with alkaline phosphatase significantly restored the suppressed ALDH2 activity to a similar level to that in corn-oil treated control. This activity change was accompanied with concurrent disappearance of acidic ALDH2 spots on 2-D gels. Since both JNK and activated (phosphorylated) JNK were translocated to mitochondria following CCl_4 exposure [39], we directly tested whether mitochondrial ALDH2 activity could be inhibited by JNK in *in vitro* experiments. Incubation of mitochondrial proteins from control rats with catalytically active JNK resulted in significant inhibition of ALDH2 activity, which was accompanied with appearance of acidic ALDH2 spots on 2-D gels. We also performed immunoblot analysis by using the anti-phospho-Ser-Pro or phospho-Thr-Pro antibody, since JNK is a proline-directed protein kinase which phosphorylates Ser or Thr followed by Pro [79]. The anti-phospho-Ser-Pro antibody recognized one band (54 kDa) in the immunoprecipitated ALDH2 protein while no band was detected with the anti-phospho-Thr-Pro antibody. These data demonstrated that Ser residue(s) of ALDH2 could be phosphorylated and thus inactivated. Based on the structural comparison and the differential effects of CCl_4 on the activities of ALDH1A1 and ALDH2, Ser^{463}-Pro of rat ALDH2 could be a major site of JNK-mediated phosphorylation [39]. All these results indicate a novel underlying mechanism by which ALDH2 can be inhibited through JNK-mediated phosphorylation, resulting in decreased cellular defense capacity and increased lipid peroxidation. These sequential events likely contribute to the pericentral necrosis in the liver [77]. Furthermore, we expect that ALDH2 could be inactivated by a similar mechanism following exposure to many other hepatotoxic agents such as acetaminophen [71] and troglitazone [78] or under many pathophysiological conditions like UV exposure where JNK is activated and translocated to mitochondria [39,80,81].

Contrary to the JNK-mediated phosphorylation and subsequent inactivation of ALDH2, other protein kinases such as protein kinase Cε (PKCε) can phosphorylate and activate mitochondrial ALDH2. In fact, administration of a small physiological and cardioprotective dose of alcohol stimulated PKCε [82], which was translocated to mitochondria in a time-dependent manner and bound ALDH2 protein [83]. Reciprocal immunoprecipitation experiments with either anti-PKCε antibody or anti-ALDH2 antibody verified that these proteins interact to each other in the mitochondria. *In vitro* incubation of ALDH2 protein with PKCε promoted phosphorylation of Thr residues of ALDH2. Mass-spectral analysis confirmed that Thr^{185}, Thr^{412} and possibly Ser^{279} of ALDH2 could be phosphorylated by PKCε, resulting in ALDH2 activation [84]. Stimulation of ALDH2 by PKCε following exposure to small doses of alcohol [82,83] or small molecule chemical activators such as

Alda-1 [84] and Alda-44 [85] correlated with decreased levels of 4-HNE and significant improvement of ischemic cardiac damage in the experimental models. Activation of ALDH2 activity through PKCε-mediated phosphorylation was also supported by the appearance of acidic ALDH2 spots on 2-D gels. By testing the effects of various known activators and inhibitors of ALDH2 including cyanamide, Mochly-Rosen and colleagues concluded that the degree of the myocardial infarct size from the experimental I/R injury inversely correlated to the ALDH2 activities [84,85]. Furthermore, the small molecule activator Alda-1 could support the suppressed activities of heterozygous *ALDH2*1/2* or homozygous *ALDH2*2/2* variants [84], suggesting that this compound and its structural analogs (including Alda-44) could help many East Asian people (> 0.5 billion people) who carry the dominant negative mutation (Glu487Lys) in the human *ALDH2* gene. Recent crystal structural analysis further described that Alda-1 supports ALDH2 activity by working as a chemical chaperone [86]. Taken together, these reports provided convincing evidence that ALDH2 can be activated by PKCε-mediated phosphorylation and that maintaining the ALDH2 activity either direct activation with small activators or indirect activation through PKCε is critically important in cardioprotection. It is of interest whether this type of PKCε-mediated protection exists in other tissues.

In addition, another report recently showed that ALDH2 could be activated through phosphatidyl inositol-3-kinase (PI3K)-dependent phosphorylation, as observed in female rats compared to male counterparts under the experimental models of cardiac I/R injury [87]. The degree of cardiac damage was less significant in females than in males, where Alda-1, a chemical activator of ALDH2 [84], exerted its beneficial effect in males but with little benefits in females. By using the fluorescence 2-D difference (2-D DIGE) proteomics approach to compare the altered levels and PTMs of cardiac mitochondrial proteins, Murphy and colleagues observed that ALDH2 were phosphorylated in greater amounts (appearance of more acidic ALDH2 spots on 2-D gels) in female rats than in males. The acidic ALDH spots, initially observed in female rat hearts, disappeared in ovariectomized female rats, suggesting a role of estrogen in ALDH2 phosphorylation and cardioprotection. Moreover, phosphorylation of ALDH2 in female rats was blocked after pre-incubation with wortmannin, a specific inhibitor of PI3K. In contrast, the effect of wortmannin on the disappearance of acidic ALDH2 spots was not observed in males. These results indicate that the PI3K-mediated phosphorylation (activation) of ALDH2 with decreased levels of 4-HNE in female rats likely contribute to cardioprotection.

Another 2-D DIGE proteomics analysis showed that glycogen-synthase kinase 3 (GSK-3) may control the expression of mitochondrial proteins since the level of an ALDH2 protein spot was slightly but significantly elevated after treatment with a specific GSK-3 inhibitor, which provides cardioprotection [88]. Based on the 2-D DIGE analysis data and no information about the ALDH activity change, it is unlikely that the expression of other ALDH2 spots, usually observed on 2-D gels, is altered under these conditions. Consequently, future studies are needed to determine which amino acids of ALDH2 can be phosphorylated by GSK-3 and how the GSK-3 mediated phosphorylation of ALDH2 affects its overall catalytic activity.

C) Nitration of ALDH2 and isozymes

Recent data showed that ALDH2 plays a central role in the biotransformation (i.e., reduction) of nitroglycerin (glyceryl trinitrate, GTN) used as an anti-ischemic drug to treat angioplasty and cardiovascular diseases [89,90]. However, chronic usage of GTN can lead to inhibition of ALDH2 activity though increased production of reactive oxygen/nitrogen species in the mitochondria and thus causes GTN tolerance (decreased reaction to GTN and decreased vasodilatation) [52,89,91]. Initial studies showed that sulfhydryl groups of ALDH2 were oxidized (to disulfides) or glutathionylated, which could be effectively

reduced to free sulfhydryl groups in the presence of DTT or dihydrolipoic acid [52,91]. Another report recently suggested that DTT could not fully restore the suppressed ALDH2 activity, suggesting partially irreversible inactivation of ALDH2 by chronic GTN usage or a bolus dose [92]. The reason for the irreversible inhibition of ALDH2 may reflect hyper-oxidation of critical Cys residues to sulfinic/sulfonic acids, which cannot be reduced by DTT [62,63]. Alternatively, ALDH2 could be inhibited through protein nitration based on the recent data where GTN promotes nitration signaling [93]. In the latter study, ALDH2 activity was mildly inhibited by NO or hydrogen peroxide in the experimental conditions, but was markedly inhibited by incubation with peroxynitrite [93]. Furthermore, in the presence of GTN, ALDH2 could serve as a peroxynitrite synthase, producing increased production of peroxynitrite, which could have nitrated Tyr residue(s) of ALDH2 [49], resulting in its inactivation. Another study provided the direct evidence for nitration of ALDH2 and isozymes. Proteomics identification followed by mass-spectral analysis confirmed that ALDH2 and ALDH7A1 were nitrated in the kidney of spontaneously hypertensive rats [94]. Thus, the potential sites of Tyr nitration(s) of ALDH2 and its implications in GTN bioactivation and cross-tolerance need to be investigated in the future.

D) Acetylation of ALDH2 and isozymes

Protein modifications by acetylation and deacetylation have emerged as an important PTM in regulating the function of many proteins especially in the mitochondria [95]. It is also known that hyper-acetylation of lysine (Lys) residues of many proteins alters their biological functions. For instance, cellular functions of many key regulatory proteins such as p53, a transcription factor, and nuclear histone proteins, involved in controlling the rate of cell growth and normal development, are regulated by acetylation and de-acetylation [96]. Some metabolic enzymes such as long-chain acyl-CoA dehydrogenase, one of the 4 enzymes in the mitochondrial fatty acid β-oxidation pathway, are also regulated by acetylation as demonstrated with the inactivation of this enzyme through hyper-acetylation of its Lys residues, as shown in mice deficient in S*irtuin 3* gene [97]. An earlier proteomics and bioinformatics study showed that ALDH2, ALDH4A1, and ALDH6A1 were acetylated under physiological conditions [98]. Recent proteomics studies revealed that many cytosolic and mitochondrial proteins are hyper-acetylated in alcohol-exposed rats [99] or high-fat exposed mice [100]. Mass-spectral analysis confirmed hyper-acetylation of many cytosolic and mitochondrial proteins including hepatic ALDH1A1 (retinal dehydrogenase), ALDH2, and ALDH3A1 (fatty aldehyde dehydrogenase) in mice exposed to a high-fat diet [100]. Since alcohol metabolism [61] or the high fat diet [100] decreases the levels of NAD^+ with diminished ratios of cellular NAD^+/NADH, hyper-acetylation of ALDH2 and ALDH isozymes seem to result from decreased activities of sirtuin proteins, which are NAD^+-dependent deacetylases [95]. However, the functional roles of acetylation of ALDH2 and other ALDH isozymes have not been studied in recent studies [99,100] and thus warrant further characterizations. Therefore, it would be of interest whether the conserved Lys residues (e.g., such as Lys^{192} and Lys^{489} in ALDH2 [101]) are a potential site of acetylation. Furthermore, it would be important to determine the functional roles of acetylation of ALDH2 and ALDH isozymes in various alcohol-related disease states since alcohol exposure decreased the activities and/or protein contents of sirtuin 1 [102,103], sirtuin 3 [104,105], and sirtuin 5 [106].

E) Formation of adducts between ALDH2 and reactive metabolites

The activities of ALDH2 and other ALDH isozymes were inhibited by exposure to many therapeutic drugs such as acetaminophen [28], daunomycin [27], cyanamide [107], daidzin [108], disulfiram [29,109], and pargyline [110]. Many toxic compounds including acrolein, bromobenzene, carbon tetrachloride, 4-HNE, MDMA (ecstasy), reserpine, and thioacetamide can also inhibit ALDH2 activity, as discussed earlier [39,40], although the

inhibitory mechanism of ALDH2 activity by each agent is poorly understood. Recent data showed that 4-HNE can inhibit the ALDH2 activity through adduct formation with the active site Cys^{302} of ALDH2 in alcohol-exposed rodents [111]. Acetaminophen, daunomycin, disulfiram, and MDMA (ecstasy) may inhibit ALDH2 activity by a similar mechanism directly through adduct formation between the critical Cys residue(s) and the reactive metabolite of each of these compounds. For instance, critical Cys residues of ALDH2 could have been conjugated with the reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinone imine, leading to its inactivation [28]. Alternatively, all these compounds inhibit ALDH2 activity indirectly through increasing the levels of peroxynitrite, which can *S*-nitrosylate Cys residues and nitrate Tyr residues [49], elevating lipid peroxides such as 4-HNE which produces an irreversible adduct [42,111], and/or promoting JNK-mediated phosphorylation of ALDH2 [39].

F) Covalent modifications and suppression of ALDH2 in pathological conditions

It is well-established that many East Asian people with facial flushing response upon alcohol consumption possess a dominant negative mutation in human *ALDH2* gene [112,113]. Some investigators proposed that *ALDH2*2* mutant gene is protective against developing alcoholism and biomedical consequences since individuals with either heterozygous *ALDH2*1/2* or homozygous *ALDH2*2/2* mutant allele do not usually drink alcohol due to flushing response and uncomfortable feeling with excessive swelling and accelerated heart rates. Biochemically, these individuals have decreased ALDH2 activities which usually result in accumulation of acetaldehyde and other lipid aldehydes [114]. These conditions with elevated levels of acetaldehyde upon alcohol intake have been simulated in experimental animal models such as *Aldh2*-null mice [115] and UChA rats [116]. In addition to ALDH2 inhibition through genetic mutations [15,18–23], ALDH2 activity or protein amount could be decreased under various pathophysiological conditions such as aged rodents [117], partial hepatectomy [118], and hepatoma [27,119], although the inhibitory mechanisms were not elucidated in each case. Global proteomics analyses followed by mass-spectral analysis identified 3 separate ALDH2 spots on 2-D gels for 10 normal tissues while only 1 most acidic ALDH2 spot was observed in 10 hepatoma tissues [119]. Similar results were also reported by another laboratory, where 11 different hepatoma tissues were analyzed [120]. Based on the 3 ALDH spots (with little change in their molecular sizes) in normal control tissues, we believe that these multiple ALDH2 spots likely represent either hyper-oxidation to sulfinic/sulfonic acids or different states of phosphorylated proteins. Interestingly, at least 3 discrete ALDH3A1 spots, a known marker of hepatic cancer [121], and 4 separate ALDH1A1 spots on 2-D gels were consistently observed in both hepatoma and normal tissues [119]. Regardless of the presence or absence of ALDH isozyme spots in hepatoma tissues, these multiple ALDH1A1, ALDH2, and ALDH3A1 spots with apparently same molecular masses on 2-D gels likely represent PTMs of each ALDH isoform, although we do not know the reason why the 2 basic ALDH2 spots disappeared in hepatoma tissues. The nature and functional role of phosphorylated ALDH2 and isozymes need to be studied.

Other amino acids of ALDH2 and isozymes could be modified by different types of PTMs. In fact, mass spectral analysis confirmed that Met residues of ALDH1A1, ALDH2, and ALDH3A1 were oxidized in hepatoma tissues [119]. Another 2-D DIGE analysis followed by mass-spectral analysis identified 5 acidic ALDH2 spots in non-small cell lung cancer tissues, suggesting multiple PTMs including glycosylation, acetylation, and phosphorylation [122]. Therefore, it is possible that other amino acids of ALDH2 can be covalently modified under different pathological conditions. However, the functional roles of these additional modifications in ALDH2 and isozymes need to be established.

G) ALDH2 as an emerging target for translational research

It is well-established that alcoholic individuals are more susceptible to many disease states [14,20,123]. This is particularly true for many East Asian people with the mutant *ALDH2* allele [20–23,113]. Chronic alcohol intake increases the levels of acetaldehyde and lipid peroxides as well as oxidative/nitrosative stress, which lowers the levels of anti-oxidants and inhibits many defensive enzymes such as superoxide dismutases, peroxiredoxin, and ALDH2, most likely through oxidative/nitrosative modifications of critical amino acids in the target proteins [32–34,58–61,124]. Consequently, chronic and excessive alcohol consumption causes alcoholic fatty liver, hepatitis, fibrosis, cirrhosis, and cancer in the liver while alcohol can also damage many other organs including the brain, heart, lung, pancreas, and testis. Furthermore, there are additive or synergistic interactions between alcohol and other potentially genetic factors/toxic substances such as hepatitis viruses, AIDS virus, obesity, diabetes, nicotine (smoking) and drugs including acetaminophen, cocaine, MDMA (ecstasy) (Figure 1). For instance, it is well-established that alcoholic individuals and people who drink alcohol regularly are more susceptible to severe liver damage caused by acetaminophen even at clinically relevant doses [125,126]. The acetamophen-related acute liver damage in alcohol-exposed subjects is believed to be promoted through alcohol-mediated induction of CYP2E1 [123], which can produce more reactive toxic metabolites, eventually contributing to mitochondrial dysfunction and apoptosis/necrosis. Additive or synergistic interactions between hepatitis viruses, diabetes, obesity, or MDMA and alcohol have been also demonstrated or observed in experimental models and human conditions [63,73,123,127–132]. Combination of these comorbidity factors significantly accelerates disease processes or aggravates the existing conditions. Therefore, many chemicals such as cyanamide [107], daidzin [108], disulfiram [109], and specific anti-sense oligonucleotides [133] or siRNAs [134] to ALDH2 have been developed to inhibit ALDH2 activity or suppress *ALDH2* gene expression. Some of these ALDH2 inhibitors such as disulfiram and cyanamide have been used in humans as deterrents for alcohol consumption to ultimately prevent alcohol-associated organ damage.

On the other hand, ALDH2 activity and/or expression are suppressed in disease states such as ischemia followed by reperfusion [37,84] and after exposure to many commonly used drugs/chemicals including acetaminophen, carbon tetrachloride, daunomycin, disulfiram, and GTN [24–29,47,52]. Due to the suppression of ALDH2 and possibly other ALDH isozymes, the levels of toxic lipid peroxidation products such as 4-HNE and MDA are likely to be elevated. Consequently, we expect increased rates of mitochondrial dysfunction and cell/organ damage. Based on the pathophysiological implications of the suppressed ALDH2 activity, it is desirable to find small molecule activators of ALDH2 or at least compounds to prevent suppression of ALDH2 to blunt accumulation of toxic aldehydes including acetaldehyde, acrolein, 4-HNE, and MDA. In fact, antioxidants such as DTT and reduced lipoic acid were effective in preventing inactivation of ALDH2, which was suppressed by GTN treatment [52,91].

Numerous reports suggest that inactive or low ALDH2 activity, through either genetic mutations or suppression by toxic compounds, seems to be a major risk factor for various disease states including alcoholic organ damage, cancer, and cardiovasucular diseases [18–28]. Therefore, it is desirable to identify potent activators of ALDH2. After high-throughput screening of a diverse library of small molecules, Mochly-Rosen and colleagues identified chemical activators of ALDH2, Alda-1 [*N*-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide] and Alda-44, both of which not only directly activate ALDH2 but also significantly protect against ischemic heart damage [84,85]. They also showed an inverse relationship between the ALDH2 activity and the degree of cardiac damage in experimental models of ischemic heart damage [84], providing convincing evidence that ALDH2 may have become a new emerging target of developing medicines for treating cardiovascular

diseases. Furthermore, they showed that Alda-1 supports the activities of over-produced recombinant ALDH2*1/2 and ALDH2*2/2 mutant proteins, whose basal activities were relatively low compared with that of ALDH2*1/1 wild-type protein. These results indicate that Alda-1 and its structural analogs including Alda-44 could be used to support many East Asian people who carry the dominant negative mutant *ALDH2*1/2* or *ALDH2*2/2* gene.

It is well-established that the efficacy and tolerance of GTN therapy in treating cardiovascular diseases depend on mitochondrial ALDH2 activity for its biotransformation. In fact, many East Asian people with a dominant negative mutation in *ALDH2*1/2* or *ALDH2*2/2* gene exhibit a decreased efficacy of GTN therapy [135]. In addition, since chronic use of GTN develops tolerance (through oxidative inactivation of ALDH2, as discussed before), it is expected that these small molecule activators of ALDH2 could be used to support the sublingual usage of GTN for treating cardiovascular diseases, especially for many East Asian people with a mutant *ALDH2* allele since these individuals cannot efficiently produce nitric oxide the active metabolite of GTN and thus have poor therapeutic benefits [135]. Alternatively, another GTN analog such as aminoethyl nitrate (AEN) [136] would be an ideal compound for treating cardiovascular disorders, since AEN does not need to be activated by ALDH2, whose activity can be inhibited through oxidative modification during GTN therapy. Furthermore, although AEN exhibited a similar potency to GTN, it neither seems to increase mitochondrial oxidative stress nor develop *in vitro* tolerance.

Based on the pathophysiological roles of ALDH2 and recent developments of drug candidates for supporting the ALDH2 activity in ischemic cardiovascular conditions, it is expected that many other small molecule activating compounds would be developed in the future. Some of the drug candidates are likely used to improve our knowledge about the roles of ALDH2 in various pathological conditions and can be used in clinics as therapeutics or supportive agents in many disease states.

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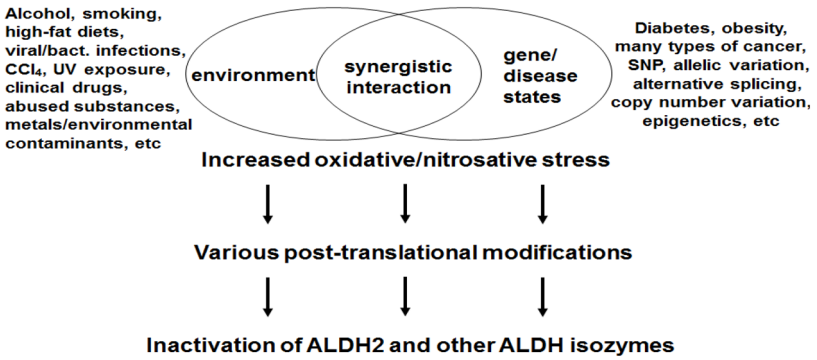

Figure 1.

Synergistic interaction between genetic and environmental factors, leading to inactivation of ALDH2 and its isozymes. Various environmental factors (including viral/bacterial infections as listed above) and genetic factors (including SNP and copy number variation) synergistically interact and increase oxidative/nitrosative stress, which promotes various PTMs, leading to inactivation of ALDH2 and other ALDH isozymes.

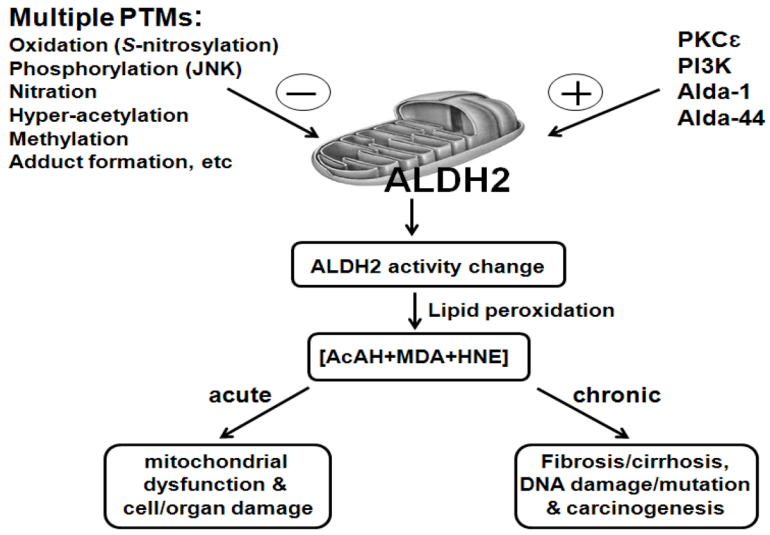

Figure 2.

Biological consequences of PTMs of ALDH2 and isozymes.

Various PTMs listed on the upper left negatively affect the ALDH2 activity, leading to increased levels of toxic aldehyde compounds. These reactive carbonyl compounds can activate the JNK- and p38 kinase-mediated cell death pathway, leading to mitochondrial dysfunction and cell/tissue damage, as recently reported [39]. They can also activate stellate cells to produce collagen and pro-fibrotic growth factors. Furthermore, the reactive carbonyl compounds can interact with mitochondrial DNA, leading to DNA damage, deletion, and mutation, ultimately contributing to carcinogenesis. In contrast, activation of ALDH2 by PKCε, PI3K or small molecule compounds Alda-1 and Alda-44 shown on the upper right can prevent accumulation of lipid peroxides and thus avoid cell/tissue damage or carcinogenesis. The negative and positive signs represent inhibition and activation of ALDH2 activity, respectively.

Table 1

Structural alignment of the region around the active site Cys residue in ALDH isozymes.

Name	Peptide Sequence	Swiss-Prot
ALDH1A1	^{275}HGV**F**YHQ**G**QC**C**I**A**ASRIFVEE^{295}	P00352
ALDH1A2	^{292}QGV**F**FNQ**G**QC**C**T**A**GSRIFVEE^{312}	O94788
ALDH1A3	^{286}QGV**F**FNQ**G**QC**C**T**A**ASRVFVEE^{306}	P47895
ALDH1B1	^{291}EAL**F**FNM**G**QC**C**C**A**GSRTFVEE^{311}	P30837
ALDH1L1	^{679}SSV**F**FNK**G**EN**C**I**A**AGRLFVED^{699}	O75891
ALDH1L2	^{700}GAV**F**FNK**G**EN**C**I**A**AGRLFVEE^{720}	Q3SY69
ALDH2	^{291}FAL**F**FNQ**G**QC**C**C**A**GSRTFVQE^{311}	P05091
ALDH3A1	^{216}WGK**F**MNS**G**QT**C**V**A**PDYILCDP^{236}	P30838
ALDH3A2	^{213}WGK**Y**MNC**G**QT**C**I**A**PDYILCEA^{233}	P51648
ALDH3B1	^{216}WFR**Y**FNA**G**QT**C**V**A**PDYVLCSP^{236}	P43353
ALDH3B2	^{135}WFC**Y**FNA**G**QT**C**V**A**PDYVLCSP^{155}	P48448
ALDH4A1	^{320}RSA**F**EYG**G**QK**C**S**A**CSRLYVPH^{340}	P30038
ALDH5A1	^{312}ASK**F**RNT**G**QT**C**VCSNQFLVQR^{332}	P51649
ALDH6A1	^{289}GAA**F**GAA**G**QR**C**M**A**LSTAVLVG^{309}	Q02252
ALDH7A1	^{302}FAAVGTA**G**QR**C**TTARRLFIHE^{322}	P49419
ALDH8A1	^{259}RSS**F**ANQ**G**EI**C**LCTSRIFVQK^{279}	Q9H2A2
ALDH9A1	^{260}MAN**F**LTQ**G**QV**C**CNGTRVFVQK^{280}	P49189

The primary amino acid sequences of the region around the active site cysteine residue of each human ALDH isozyme, as listed in Marchitti et al [10], are aligned except for ALDH16A1 and ALDH18A1, since the active site Cys residues in the latter 2 enzymes are not conserved. The active site Cys (C) and other highly conserved amino acids are presented in bold characters.