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Malondialdehyde inhibits an AMPK-mediated nuclear translocation and repression activity of ALDH2 in transcription

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ABSTRACT

Aging process results from deleterious damages by reactive oxygen species, in particular, various metabolic aldehydes. Aldehyde dehydrogenase 2 (ALDH2) is one of metabolic enzymes detoxifying various aldehydes under oxidative conditions. AMP-activated protein kinase (AMPK) plays a key role in controlling metabolic process. However, little was known about the relationship of ALDH2 with AMPK under oxidative conditions. Here, we, by using MDA-specific monoclonal antibody, screened the tissues of young and old rats for MDA-modified proteins and identified an ALDH2 as a prominent MDA-modified protein band in the old rat kidney tissue. ALDH2 associates with AMPK and is phosphorylated by AMPK. In addition, AICAR, an activator of AMP-activated protein kinase, induces the nuclear translocation of ALDH2. ALDH2 in nucleus is involved in general transcription repression by association with histone deacetylases. Furthermore, MDA modification inhibited the translocation of ALDH2 and the association with AMPK, and ultimately led to de-repression of transcription in the reporter system analysis. In this study, we have demonstrated that ALDH2 acts as a transcriptional repressor in response to AMPK activation, and MDA modifies ALDH2 and inhibits repressive activity of ALDH2 in general transcription. We thus suggest that increasing amount of MDA during aging process may interrupt the nuclear function of ALDH2, modulated by AMPK.

1. Introduction

The oxidative stress theory of aging implicates that aging results from random deleterious damages to tissues either by reactive oxygen or reactive nitrogen species [1]. In this context, we have paid a special attention in looking for the link between oxidative stress and genetic control for aging phenomenon. Polyunsaturated fatty acids are readily oxidized to a broad spectrum of reactive compounds such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) [2]. Since MDA has the ability to crosslink biomolecules by binding to two molecules at the same time, MDA modification might be expected to contribute to functional deterioration in aging process. Therefore, identification of MDA-modified proteins *in vivo* tissues from the aging organisms would be prerequisite to understanding aging mechanism.

Aldehyde dehydrogenase 2 (ALDH2) is a metabolic enzyme which detoxifies various oxidative aldehydes, expressed in various tissues with its highest level in liver [3]. Since ALDH2 has a high activity especially for oxidation of acetaldehyde, it plays a major role in alcohol detoxification [4]. However, absence of any significant adverse developmental or physiological problems in homozygous variant subjects with null ALDH2 activity suggests that ALDH2 might be either non-essential for survival or well compensated by other related enzymes [5]. Nonetheless, it would be pertinent to assume that its functional changes might substantially influence the organism's sensitivity to deleterious effects of oxidative stress especially of a variety of aldehyde attacks in metabolic overload. Aldehyde oxidation and its conjugation with glutathione are suppressed during aging [6], implementing the age-dependent functional changes of ALDH2. Therefore, a novel role of ALDH2, which might be affected by MDA modification, was conjectured.

AMP-activated protein kinase (AMPK) plays a key role in sensing intracellular ATP levels and maintaining cellular energy balance [7]. Once activated, AMPK phosphorylates several downstream substrates to switch off ATP-consuming pathways and to switch on ATP-generating pathways [8–11]. Moreover, activation

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of AMPK has long-term effects of altering both gene expression [12–14] and protein synthesis [15,16].

As ALDH2 is a well-known metabolic enzyme responsible for detoxification of metabolic aldehydes, it would be reasonable to assume that it could be regulated by a variety of metabolic pathways, especially by AMPK, the energy sensor. Nonetheless, very little has been known about the relationship of ALDH2 with AMPK. In this study, we first identified ALDH2 as an MDA-modified protein in old rat kidney tissue by MALDI-TOF mass spectroscopy. We found that ALDH2 could move from mitochondria into nucleus by AMPK activation. Furthermore, nuclear ALDH2 serves as a general transcriptional repressor. However, MDA-modified ALDH2 lost its binding to AMPK and translocation into nucleus, and ultimately led to loss of transcriptional repressive activity. Therefore we suggest that increasing amount of MDA during aging process may interrupt the nuclear function of ALDH2, modulated by AMPK.

2. Materials and methods

2.1. Antibodies

Anti-flag and anti-β-actin antibody were purchased from Sigma–Aldrich; anti-myc and anti-hemagglutinin (HA) antibody from Covance (Berkeley, CA); peroxidase conjugated anti-rabbit IgG antibody and peroxidase conjugated anti-mouse IgG antibody from Pierce; other antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). All fluorescence dye-conjugated antibodies were purchased from Molecular Probes (Eugene, OR). Anti-ALDH2 monoclonal antibody was prepared by Metabolic Engineering Laboratories (Seoul, Korea). Anti-MDA monoclonal antibody was prepared as described previously [17].

2.2. Animals

Harlan Sprague–Dawley (SD) rats were purchased and maintained from the Animal Laboratory of Seoul National University College of Medicine. Old animals were maintained on a normal diet for more than 22 month. 4–6-week-old female rats were used as young controls. All animals were handled in accordance with the guidelines of the Korea Food and Drug Administration.

2.3. Cell culture and transfection

HEK293 cell was purchased from ATCC (Manassas, VA). Cells were cultured in DMEM medium containing 10% (v/v) fetal bovine serum and 50 U/ml of streptomycin and penicillin. Cells were transfected with the indicated plasmids using LipofectAMINE PLUS™ according to the manufacturer's protocol. DNA construct of pT7-7-ALDH2 was obtained from Dr. Weiner, H. (Purdue University), while mammalian expression vector pcDNA™ series and pSG5-HA from Invitrogen and Stratagene and bacterial expression vector pET-15b from Novagen.

2.4. Cell lysis and immunoprecipitation

Cells were lysed with lysis buffer [20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 0.5% (v/v) IGEPAL® CA-630, 1 mM EDTA), 1 mM PMSF]. Whole cell lysates were incubated with indicated antibodies along with Protein (A/G)-agarose beads (Santa Cruz Biotechnology) for 4 h. The immunocomplexes were washed three times with lysis buffer and then subjected to SDS–PAGE.

2.5. Knockdown of ALDH2 by RNA interference

The ALDH2 siRNA used in this study was purchased from Santa Cruz Biotechnology. The cells were transfected with ALDH2 siRNA using the Oligofectamine reagent (Invitrogen). After 72 h, cells were harvested and lysed with lysis buffer. The lysates were then subjected to SDS–PAGE and immunoblotted with anti-ALDH2 antibody.

2.6. Immunostaining and immunohistochemical staining

Cells were placed on glass coverslips in 6-well plate and fixed with 4% (w/v) paraformaldehyde in PBS. Cells were permeabilized with 0.5% (v/v) triton X-100 in PBS and blocked with 2% (w/v) bovine serum albumin in PBS. Then cells were incubated with anti-myc antibody for 1 h and washed three times with PBS. After incubation with FITC-conjugated secondary antibody for 1 h, cells were stained with DAPI and visualized with confocal laser scanned microscopy (Bio-Rad). For immunohistochemical staining, rat tissues were fixed, paraffin embedded, placed for peroxidase immunohistochemical staining by Vector stain Elite ABC kit (Vector Laboratories).

2.7. Transport of recombinant proteins into the cells

cDNAs of GAL4 DNA binding domain-ALDH2-myc and ALDH2-myc were cloned into pET15b vector and transformed to BL21(DE3) (Invitrogen) strain of *Escherichia coli*. Expression of this gene was carried out at 37 °C by IPTG of final concentration of 1 mM for 4 h. Cells were collected, and expressed proteins were purified in denatured condition using TALON® Metal Affinity Resin (Clontech) according to the manufacturer's instruction. 100 nM of bacterially purified ALDH2-myc was reacted with 200 nM MDA at 37 °C for 1 h. Then ALDH2-myc was mixed with Tranport™ Protein Delivery Reagent (Cambrex) at room temperature for 30 min. Resulting mixture was added into the medium, and cells were incubated for 2 h.

2.8. Identification of MDA-modified protein in the tissues of old rat kidney

Purification scheme for identification of MDA-modified protein was shown in Supplementary Fig. 1. In brief, tissues were homogenized with Polytron® (Fisher Scientific). After centrifugation, supernatants were precipitated by adding ammonium sulfate to 50% (w/v) saturation solution. Pellet was collected and dissolved and dialyzed. Samples were affinity-purified with anti-MDA antibody plus protein G-agarose. An excess of blood-born antibodies was removed by adding more of protein G-agarose to purified solution. Finally the supernatants were applied to SDS–PAGE, and resolved by silver staining. The resulting bands were identified by MALDI-TOF mass spectroscopy.

2.9. In vitro kinase assay

The active AMPK holo enzyme was purchased from Millipore (Cat. #14–840). Twenty nanogram of AMPK and bacterially purified $(His)_6$-ALDH2 were incubated in kinase assay buffer (8 mM MOPS-NaOH, pH7.0, 100 μM sodium orthovanadate, 5 mM DTT, 5 mM β-glycerophosphate, 10 mM $MgCl_2$, 250 μM AMP, 200 μM ATP) with 10 μCi γ-[^{32}P]ATP at 30 °C for 30 min. The reaction was terminated by adding 5× sample buffer and then subjected to SDS–PAGE. The phosphorylation of ALDH2 was detected by autoradiography.

2.10. Statistics

Data are presented as means ± standard error (SEM.), and p values were calculated using Student's t test. A value of $p < 0.05$ was

considered statistically significant. All data presented are representative of at least three separate experiments.

3. Results

3.1. ALDH2 was identified as an MDA-conjugated protein in old rat kidney

By using MDA-specific monoclonal antibody, we screened a variety of tissues from young and old rats by immunoblot analysis [17]. And we found that old kidney tissue has a strong MDA-modified protein band with 55 kD of molecular weight in contrast to its absence in young kidney (Fig. 1A). Immunohistochemical study illustrated that old kidney, but not young kidney, was strongly reacted to the anti-MDA monoclonal antibody (Fig. 1B), especially at glomeruli.

To identify MDA-modified protein in old rat kidney, we partially purified the protein according to a scheme shown in Fig. S1. We identified this MDA-modified protein as an ALDH2 (Table. S1). To confirm this identification of MDA-modified ALDH2 *in vivo*, kidney tissue homogenates were initially precipitated with anti-ALDH2 monoclonal antibody and then checked with anti-MDA antibody. As expected, ALDH2 from old rat kidney was more heavily modified by MDA than that from young kidney (Fig. 1C), confirming that the major MDA-modified protein in old kidney would be ALDH2. These data imply that MDA-mediated modification of ALDH2 may cause the change of its function under oxidative conditions in aging process.

3.2. MDA interrupts the interaction between ALDH2 and AMPK

Meantime, AMPK is known to regulate metabolic pathways by phosphorylating important metabolic enzymes [18]. Based on our finding that ALDH2 is a metabolic enzyme which is modified by MDA in old rat tissue, we speculated whether AMPK would control the function of ALDH2.

To address this question, we first examined their binding by transfection of ectopically expressed HA-AMPK and ALDH2-myc into HEK293 cells followed by immunoprecipitation of cell lysates with anti-HA antibody and probed with anti-myc antibody or vice versa. Both of immunoprecipitation experiments showed the interaction of ALDH2 with AMPK (Fig. 2A). As AMPK can phosphorylate important metabolic enzymes, we examined the phosphorylation of ALDH2 by AMPK. An active AMPK was incubated with bacterially-purified $(His)_6$-ALDH2 in presence of γ-[^{32}P]ATP. As expected, ALDH2 was phosphorylated by AMPK (Fig. 2B), indicating that ALDH2 is a substrate of AMPK. In addition, we confirmed that immunoprecipitates of anti-MDA monoclonal antibody specifically contained ALDH2 treated with MDA in HEK293 cells (Fig. 2C), indicating that MDA could modify ALDH2 in HEK293 cells.

To test the effect of MDA on this interaction, we first treated MDA with bacterially-purified ALDH2-myc and incubated it with cell lysates containing ectopically expressed HA-AMPK. Mixtures were precipitated with anti-HA antibody and probed with anti-myc antibody (Fig. 2D). Interestingly, we found that MDA-modified ALDH2 failed to interact with AMPK, indicating that MDA-modified ALDH2 might lose binding activity with AMPK.

3.3. MDA blocks the AICAR-mediated nuclear localization of ALDH2

Before we investigated the localization of ALDH2, we first tested the knockdown of ALDH2 using RNA interference. The treatment of small interference RNA (siRNA) targeting ALDH2 with HEK293 cells significantly reduced the expression of ALDH2 protein (Fig. 3A). In the same experimental conditions, we examined the localization of ALDH2 using confocal microscopy. We found that AICAR treatment triggers the translocation of ALDH2 into nucleus (Fig. 3B). Knockdown of ALDH2 led to the disappearance of ALDH2, confirming that the stained protein is ALDH2 indeed.

To investigate the effect of AICAR on localization of ALDH2, HEK293 cells were transiently transfected with myc-tagged ALDH2 and treated with AICAR. We then made cellular fraction using

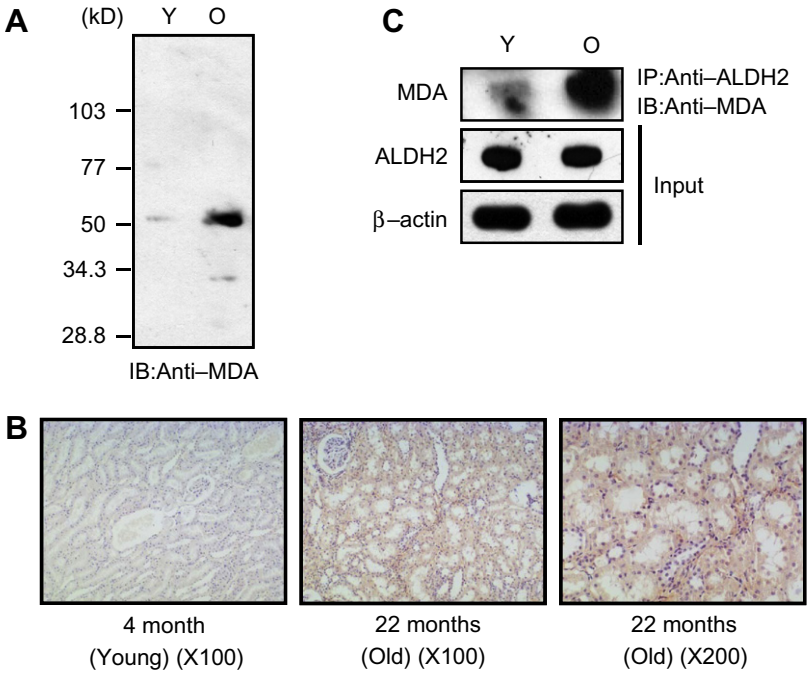

Fig. 1. MDA modified ALDH2 in old rat kidney. (A) The whole tissue lysates of young and old rat kidney were loaded onto SDS–PAGE, and then immunoblotted by anti-MDA monoclonal antibody. Y, young rat (4-month-old); O, old rat (22-month-old). (B) Immunohistochemistry of young and old kidney tissues with anti-MDA monoclonal antibody. The magnification is shown under the figure. (C) MDA-modified ALDH2 in young and old rat kidney tissues. The lysates were immunoprecipitated with anti-ALDH2 mAb and probed with anti-MDA mAb.

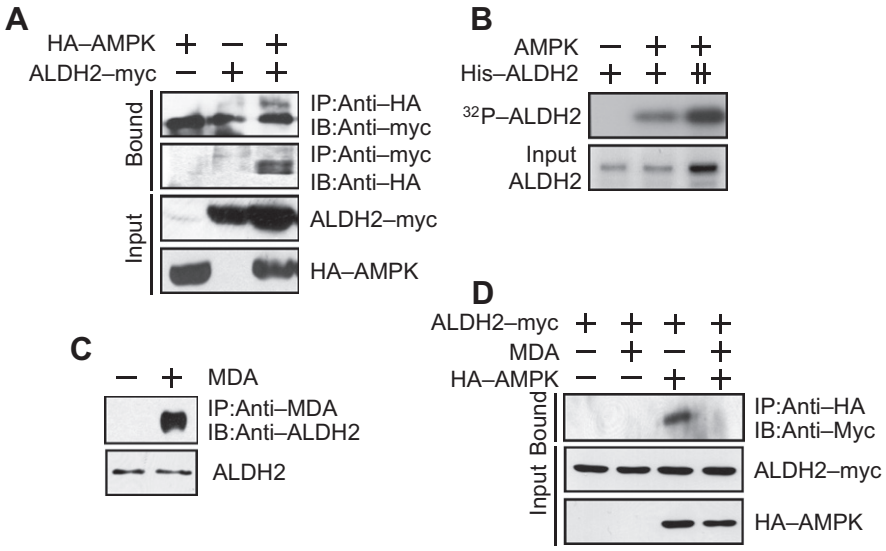

Fig. 2. MDA interrupts the interaction between ALDH2 and AMPK. (A) Interaction between ALDH2 and AMPK. HA-AMPK and ALDH2-myc were co-transfected into HEK293 cells. (B) AMPK phosphorylates ALDH2. Recombinant catalytically-active AMPK was incubated with bacterially-purified $(His)_6$-ALDH2 in presence of $\gamma[^{32}P]ATP$. (C) MDA modified ALDH2 in HEK293 cells. Cells were treated with MDA. Cell lysates were immunoprecipitated with anti-MDA antibody and probed with anti-ALDH2 antibody. (D) MDA-modified ALDH2 could not bind to AMPK. Cell extracts containing HA-AMPK were mixed with either MDA-modified ALDH2-myc or unmodified ALDH2-myc. Then AMPK was precipitated using anti-HA antibody and Protein G-agarose bead. Beads were washed three times with the lysis buffer and analyzed by immunoblot with anti-myc antibody.

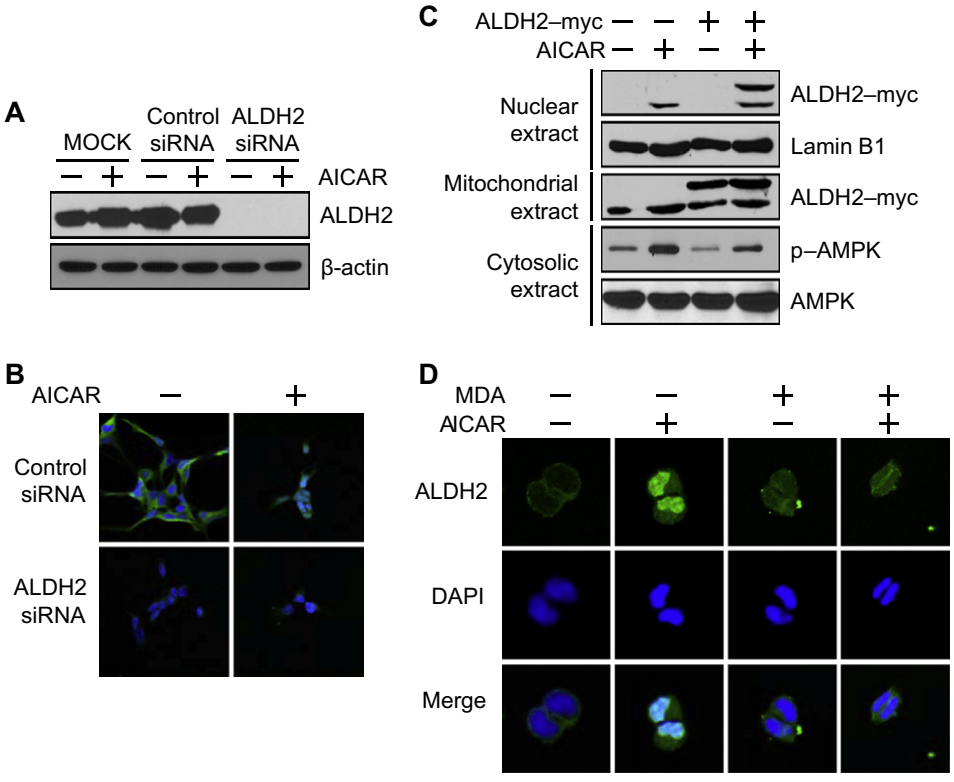

Fig. 3. MDA blocks the AICAR-mediated nuclear localization of ALDH2. (A) ALDH2 protein level is decreased by treatment of ALDH2 siRNA. ALDH2 siRNA and control siRNA were transfected into HEK293 cells, and the protein levels of ALDH2 and β-actin were analyzed by immunoblot. (B) Translocation of ALDH2 to nucleus was visualized only when control siRNA was treated into the cells. siRNA-treated cells were incubated with 2 mM AICAR for 2 h. Cells were stained with anti-myc antibody (Green). The nucleus was stained by DAPI (Blue). (C) Translocation of ALDH2 from the mitochondria to the nucleus. HEK293 cells were transfected with plasmid encoding ALDH2-myc. After 48 h, 2 mM of AICAR was treated into the cells for 2 h. Cells were harvested and fractionated to the nucleus, the mitochondria and the cytosol. Resulting fractions were analyzed by immunoblot with the corresponding antibodies. (D) MDA-modified ALDH2 was not translocated into the nucleus by AICAR. Bacterially purified ALDH2-myc that was *in vitro* modified by MDA was transfected into the cells. In the treatment of AICAR, localization of ALDH2 in the cells was visualized by anti-myc antibody (Green). The nucleus was stained with DAPI (blue).

centrifugation and followed by immunoblot analysis. An ectopically expressed ALDH2 was found in mitochondrial fraction regardless of AICAR-treatment. However, ALDH2 was specifically detected in nucleus when AICAR was treated (Fig. 3C), indicating that AMPK activation by AICAR triggered the translocation of ALDH2 from mitochondria into nucleus.

ALDH2 is highly MDA-modified in the old rat kidney with age-dependent manner (Fig. 1). Therefore, for the further question on the relation between the role of ALDH2 in transcriptional regulation and its modification by MDA, purified recombinant myc-tagged ALDH2 and its MDA-modified form *in vitro* were transfected into the cells in the presence or absence of AICAR. In consequence, ALDH2, not MDA-modified ALDH2, was observed to be translocated into the nucleus by AICAR (Fig. 3D), which is consistent with the fact that MDA interrupted the binding between ALDH2 and AMPK (Fig. 2D). This is the first demonstration that ALDH2 can translocate into the nucleus by AMPK activation.

3.4. ALDH2 is a general transcription repressor via binding to HDACs

To analyze the function of ALDH2 in nucleus, GAL4 DNA binding domain (DBD)-fused ALDH2 plasmid was transfected into HEK293 cells in a concentration-dependent manner. Interestingly, the relative luciferase activity was decreased in proportion to the increase of transfected plasmid (Fig. 4A), implying the repressive activity of ALDH2 on transcriptional machinery.

For the mechanism of transcriptional repression by ALDH2, we tested whether ALDH2 could bind to histone deacetylase (HDAC) family, a general epigenetic transcriptional repressor family. Immunoprecipitation test showed that ALDH2 was bound to HDAC1, HDAC2 and HDAC3, respectively (Fig. 4B), implicating the transcriptional repression function by binding of ALDH2 with HDAC complex. Binding of ALDH2 to HDACs resulted in activation of histone deacetylase activity. Nuclear extracts from AICAR-treated cells had higher histone deacetylase activity than those from untreated cells (Fig. 4C). In short, these evidences of ALDH2 translocation into nucleus by AICAR (Fig. 3), its binding to HDACs (Fig. 4B) and the resulting higher HDAC activity (Fig. 4C) suggest that ALDH2 would play an epigenetic regulatory role in gene expression through modulation of HDAC activity.

For the analysis of MDA effect on transcriptional control, we performed reporter gene assay using the repression system treated with MDA in a concentration-dependent manner. Previous data showed that Gal4-ALDH2 repressed the transcriptional activity of reporter gene, but the degree of repression was reduced in the presence of MDA (Fig. 4D). This suggests that MDA might interfere with the transcriptional repression activity of ALDH2. When the binding to AMPK was compared, MDA-modified ALDH2 could not bind to AMPK in contrast with the ready binding of wild type (Fig. 2D). MDA modification of ALDH2 might interrupt its binding to AMPK, resulting in the loss of translocation of ALDH2 into the nucleus. Activated AMPK binds to ALDH2, leading to its translocation into nucleus, where ALDH2 enhances the histone deacetylase activity by interacting with HDACs, resulting in transcriptional repression.

4. Discussion

We in this study identified ALDH2 as an MDA-modified protein in old rat kidney tissue by MALDI-TOF mass spectroscopy. ALDH2 is expressed ubiquitously but in high levels in liver, kidney, and heart due to transactivation by hepatic nuclear factor 4 (HNF4) [19]. However, any apparent MDA-modified ALDH2 in liver and heart was not detected, implicating the active and efficient radical scavenging system in those organs in contrast to functional inefficiency of aged kidney to overcome the oxidative damages. The abundance of MDA-modified ALDH2 in the aged kidney might be expected from the organ's nature of collecting and disposal of metabolic waste products, premising the higher probability of oxi-

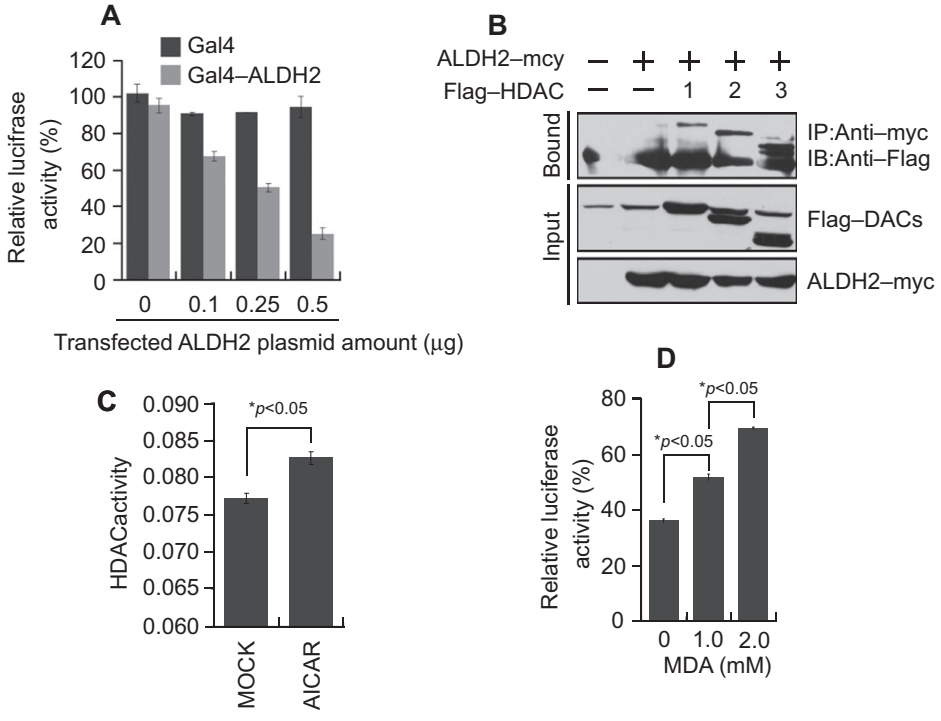

Fig. 4. ALDH2 is a general transcription repressor via binding to HDACs. (A) ALDH2 as a transcriptional repressor. pGAL4-tk-luc and GAL4-fused ALDH2 were transfected into HEK293 cells. After 48 h, the luciferase activity was measured by luminometer. (B) ALDH2 is bound to HDACs. Flag-HDACs and ALDH2-myc were transfected into HEK293 cells. Cell lysates were immunoprecipitated with anti-myc antibody and probed with anti-flag antibody (C) Relative HDAC activity of anti-ALDH2-immunoprecipitates in the presence or absence of AICAR. $^*p < 0.05$ by t test. Error bars indicate SEM. ($n = 3$). (D) The transcriptional repression activity in the presence of MDA. pGAL4-tk-luc and GLA4-fused ALDH2 were transfected into HEK293 cells, and then MDA was treated in the medium in a dose-dependent manner. The repression activity was measured by luminometer. $^*p < 0.05$. Error bars indicate SEM. ($n = 3$).

dative damages in the kidney tissue by aging. Moreover, high level of MDA-modified proteins could be observed in the glomerular capillary endothelial cells implementing the aging-dependent deteriorated renal physiology.

Monitoring the cellular location of ALDH2 after AMPK activation with AICAR showed its presence in the nucleus (Fig. 3), implying the nuclear translocation of the mitochondrial ALDH2.

Since regulation of gene expression takes place in the nucleus, the nuclear translocation of ALDH2 might implicate its certain role in regulation of transcriptional machinery. GAL4 system was utilized for ALDH2 effect study on epigenetic control. Only in the repression system, the relative luciferase activities decreased with increase of transfected plasmid (Fig. 4A), connoting the repressive activity of ALDH2 on transcriptional machinery.

To explain the transcriptional repression mode, interaction of ALDH2 with histone deacetylase (HDAC) family was monitored, resulting in binding of ALDH2 with HDAC1, HDAC2 and HDAC3, respectively (Fig. 4B). Transcriptional repression of ALDH2 might be achieved by binding to HDAC complex. Binding of ALDH2 with HDAC complex actually activated HDAC activity (Fig. 4C). These evidences of ALDH2 translocation into nucleus by AICAR (Fig. 3), its binding to HDACs (Fig. 4B) and the resulting higher HDAC activity (Fig. 4C) strongly suggest that ALDH2 would play an epigenetic regulatory role through modulation of HDAC activity. Therefore, this work shows for the first time that ALDH2 might play as one of the general repressors for transcriptional control by nuclear translocation by AMPK activation.

ALDH2 is highly MDA-modified in the rat kidney with age-dependent manner. Thereby, we asked the further question in effects of MDA modification on the role of ALDH2 in transcriptional regulation. Only the free ALDH2 could translocate into nucleus by AICAR treatment, while MDA-modified one could not (Fig. 3D). MDA modification of ALDH2 reduced the degree of repression in transcriptional control (Fig. 4D). The failure of MDA-modified ALDH2 for binding to AMPK in contrast to ready binding of wild type (Fig. 2D) results in loss of translocation capacity of ALDH2 into the nucleus. Activated AMPK binds to ALDH2, leading to its translocation into nucleus, where ALDH2 enhances the histone deacetylase activity by interacting with HDACs, resulting in transcriptional repression. These whole processes can be adjusted simply by MDA status of ALDH2, blocking its nuclear translocation, indicating the functional linkage of ALDH2 with oxidative stress condition in the regulation of global gene expression. This ALDH2-dependent transcription modulation might provide a new clue for explanation of the common cellular behaviors toward oxidative damages despite their random nature of damages.

We found that ALDH2 belongs to one of metabolic enzymes serving as transcriptional regulator. Similar dehydrogenases such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and malate dehydrogenase-1 (MDH1) have the epigenetic transcriptional activities, but these are cytosolic proteins [20–23]. ALDH2 is a mitochondrial protein with a signal sequence for mitochondrial import [24]. Mitochondrial proteins are not easily translocated to the nucleus although a few exceptions are known. For example, apoptosis-inducing factor (AIF) is translocated from the mitochondria into the nucleus in the apoptosis process [25,26]. Therefore, ALDH2 is another probable candidate as a nuclear translocating enzyme from mitochondria.

Taken together, our finding raises the possibility that ALDH2 as the transcriptional regulator might function as a direct sensor of the cellular metabolic state for regulating gene expression at general transcription level. It will be interesting to work the detailed mechanism by which ALDH2 regulates the global gene expression under oxidative stress conditions such as aging process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.131.

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