A Novel Suppressive Effect of Alcohol Dehydrogenase 5 in Neuronal Differentiation*

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Background: The role of ADH5 in neuronal development and differentiation remains unknown.

Results: ADH5 denitrosated HDAC2 and thus negatively regulates neurite growth of hippocampal neurons and neuronal differentiation of hNSCs.

Conclusion: ADH5 is a novel suppressor of neuronal differentiation.

Significance: These results advance our understanding of the role of ADH5 in neuronal differentiation.

Alcohol dehydrogenase 5 (ADH5) is a conserved enzyme for alcohol and aldehyde metabolism in mammals. Despite dynamic expression throughout neurogenesis, its role in neuronal development remains unknown. Here we present the first evidence that ADH5 is a negative regulator of neuronal differentiation. Gene expression analyses identify a constant reduction of ADH5 levels throughout neuronal development. Overexpression of ADH5 reduces both development and adult neuronal differentiation of mouse neurons. This effect depends on the catalytic activity of ADH5 and involves ADH5-mediated denitrosation of histone deacetylase 2 (HDAC2). Our results indicate that ADH5 counteracts neuronal differentiation of human neural stem cells and that this effect can be reversed by pharmacological inhibition of ADH5. Based on these observations, we propose that ADH5 is a novel suppressor of neuronal differentiation and maturation. Inhibition of ADH5 may improve adult neurogenesis in a physiological or pathological setting.

Neuronal differentiation is an important process in which neuronal precursor cells develop into functional mature neurons; it includes the projection of neuronal axons and dendrites, connection of synapses, and eventual formation of neuronal circuits. The process of neuronal differentiation (or “neurogenesis”) in mammals is highly regulated and is coordinately controlled by a complicated signaling and transcriptional network. This process is dynamically regulated by different layers of molecular switches, including protein post-translational modifications (1–4). Manipulation of kinase, transcription factor, and epigenetic modifiers has been shown to regulate neuronal differentiation (5–7). However, little is known on whether the enzymes involved in cell metabolism regulate this process.

ADH5, a well conserved enzyme from bacteria to human, is involved in the metabolism of alcohols and aldehydes in mammalian cells (8–11). Unlike other members of the ADH family with tissue-specific patterns of expression, ADH5 is ubiquitously expressed in both embryonic and adult tissues, suggesting a housekeeping function of this protein (12, 13). An important function of ADH5 in development has been reported in Drosophila and mouse. Deletion of a conserved negative regulatory element in the Drosophila adh gene led to developmental retardation in larval stages (14). Deficiency of adh5 impaired the development from lymphoid progenitors to either T or B lymphocytes in mouse (15). ADH5 is the only ADH member present in the rodent brain, and it has been found by in situ hybridization that adh5 mRNA was significantly higher in the developing rat brain from embryonic day 12.5 (E12.5) to E18.5 than that in postnatal rat brain (12, 16). However, the role of ADH5 in regulating mammalian neurogenesis remains unknown. Here we demonstrated that the expression of ADH5 was down-regulated during neuronal differentiation and neurite outgrowth. Importantly, decreased ADH5 expression was required for the commitment to neuronal lineages, whereas overexpression of ADH5 blocked neurogenesis. The role of ADH5 in antagonizing neuronal differentiation was dependent on its catalytic activity, and an ADH5-mediated denitrosation of HDAC2 mechanism was involved in this process. Finally, a suppressive effect of ADH5 in counteracting neuronal differentiation was observed in the context of differentiation of human neural stem cells into neurons, and pharmacological inhibition of ADH5 activity reversed this suppression.

The abbreviations used are: ADH5, alcohol dehydrogenase 5; DIV, days in vitro; E, embryonic day; HDAC2, histone deacetylase 2; hNSC, human neural stem cells; MAP2, microtubule-associated protein 2; GAD67, glutamic acid decarboxylase 67; qPCR, quantitative PCR; OB, olfactory bulb; EGFP, enhanced green fluorescent protein; mt, mutant; SVZ, subventricular zone; RMS, rostral migratory stream.
EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Regents—The following antibodies were used: β-actin (Santa Cruz Biotechnology), HDAC2 (Santa Cruz Biotechnology), ADH5 (Proteintech), Sox2 (Abcam), Pax6 (Vector Laboratories), Nestin (Millipore), Kif6 (Covance), MAP2 (Millipore), and Tuj1 (Sigma-Aldrich). ADH5 inhibitor C3 was purchased from ChemDiv Inc. cDNA fragments encoding ADH5 were amplified by PCR using human cDNA as a template. ADH5-encoding cDNA fragments were cloned into the vectors EGFP-N2 and pcDNA3.1, respectively. Site-directed mutants of ADH5 (R115D) were constructed using the Easy Mutagenesis System (Beijing TransGen Biotech) according to the manufacturer’s protocol, which resulted in the missense mutation of arginine 115 to aspartic acid. The ADH5 shRNA plasmid (sh-ADH5) for rat was constructed and tested in our previous work (17). pME18S-HDAC2 was kindly provided by Dr. Edward Seto (H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, FL), and the mutant of HDAC2 (HDAC2 (C262A/C274A)) was generated using the Easy Mutagenesis System as described previously (7). DNA sequences of all clones were confirmed by DNA sequencing. All chemical reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Mice—ADH5−/− mice were generated as described (13) and compared with age-matched wild-type (WT) C57BL/6. All mice were bred in the specific pathogen-free barrier facility of the Institute of Biophysics, Chinese Academy of Science.

Cell Culture and Transfection—293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Hippocampal neurons from E18 Sprague-Dawley rat or C57 BL/6 embryos were prepared as described previously (19). Nucleofector system (Amazax Biosystems) and Lipofectamine 2000 (Invitrogen) were used for DNA transfection following the instructions provided by the manufacturer.

Morphological Analysis of Mouse Hippocampal Neurons in Vitro and in Vivo—For the longest neurite analysis, hippocampal neurons via nucleofection were fixed with 4% paraformaldehyde at 2 days in vitro (DIV2). Photos were taken of at least 40 neurons selected randomly per group using an inverted fluorescence microscope (CF Plan 20×/0.4 NA objective lens; ECLIPSE Ti-E; Nikon) in conjunction with ECLIPSE Ti-E acquisition software (Nikon) and then traced and analyzed with NeuronJ (20). Primary hippocampal neurons from WT or ADH5−/− mice were transfected with YFP at DIV6 for 1 day and randomly selected by YFP expression. These neurons were photographed using the FluoView FV1000 laser scanning confocal imaging system (PLAPO 60×/1.42 NA oil objective lens; Olympus) in conjunction with FluoView FV1000 acquisition software (Olympus). All processes and their branches were traced by NeuronJ and analyzed by Sholl analysis as described previously (21). The morphology of hippocampal neurons in vivo was determined as described previously (22). Neurotrophin-stained neurons were acquired with the FluoView FV1000 laser scanning confocal imaging system (UPLSAPO 20×/0.75 NA; Olympus) in conjunction with the FluoView FV1000 acquisition software (Olympus). All processes and their branches were traced by NeuronJ and analyzed by Sholl analysis as described previously (21). Quantifications of the number of the secondary basal dendrites (branches from primary dendrites) and the process terminals per pyramidal neuron were assessed.

Generation and Culture of hNSCs—Human neural stem cells (hNSCs) were differentiated from H9 human embryonic stem cells, according to our previous study (23). hNSCs were maintained on Matrigel (BD Biosciences) in NSC medium containing 50% Advanced DMEM/F12 (Life Technologies), 50% Neurobasal medium (Life Technologies), 1× N-2 supplement (Life Technologies), 1× B-27 supplement (Life Technologies), 2 mM GlutaMAX (Life Technologies), 10 ng/ml human leukemia inhibitory factor (Millipore), 2 μM SB431542 (Tocris Bioscience), and 3 μM CHIR99021 (Tocris Bioscience). Medium was changed every day, and cells were passaged upon 80–100% confluence.

Differentiation of hNSCs toward Neurons—Spontaneous neuronal differentiation from hNSCs was performed as described previously (23).

Lentivirus Production—For generating lentiviral vectors, cDNAs from WT and mutant ADH5 were amplified by PCR and cloned into pLHE4 lentiviral vector (a gift from Dr. Tomoaki Hishida). HEK293T cells were transfected with a lentiviral vector together with packaging plasmids pMD2.G and pVSVG (Addgene). Supernatants containing virus were harvested at 48 and 72 h after transfection and filtered with a 0.45-μm PVDF membrane (Millipore).

Transwell Migration Assay—Assay was performed with Transwell permeable supports (Corning Costar). In short, first each well was coated with Matrigel at 37 °C for 1 h, and subsequently, hNSC culture medium was added into each well. Lentivirus-infected hNSCs (2×10^4 cells suspended in basic medium containing 50% Advanced DMEM/F12, 50% Neurobasal medium) were seeded to the top chamber, and then the top chambers were placed into the wells mentioned above followed by culturing at 37 °C with 5% CO2. Cells in the top chamber were fixed with 4% paraformaldehyde 48 h after incubation and stained with 0.1% crystal violet. Finally, the number of migrated cells was counted with light microscope.

Quantitative Real-time PCR—Total RNA was extracted with TRIzol reagent (Life Technologies) and used to perform reverse transcription by TransGen transcript first-strand cDNA synthesis supermix (TransGen Biotech) according to the manufacturer’s protocols. Quantitative PCR was performed with the GoTaq® qPCR master mix (Promega) by the CFX384™ real-time PCR detection system (Bio-Rad), with β-actin or GAPDH as an internal control.

Western Blot Analysis—Protein extracts from cells and tissues were separated by 10% SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was treated with 5% (w/v) fat-free milk in TBS containing 0.05% Tween 20 for 2 h and incubated with the indicated antibody for 2 h followed by incubation with peroxidase-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology) for 2 h. The epitopes were visualized with an ECL Western blot detection kit (Pierce).
Biotin Switch Assay—The biotin switch assay for detecting S-nitrosation was carried out as described previously with minor modifications (24).

Immunofluorescence Staining—Cells were fixed by 4% formaldehyde in PBS for 30 min at room temperature and subsequently treated with 0.4% Triton X-100 in PBS for 10 min at room temperature. Cells were blocked with 10% donkey serum in PBS for 1 h at room temperature and then incubated with primary antibody at 4 °C overnight. Cells were washed in PBS and incubated with the corresponding secondary antibody for
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1 h at room temperature. DNA was stained with Hoechst 33342 (Life Technologies). The microscopy was performed using the TCS SP5 II laser scanning confocal imaging system (HC PL FLUOTAR 10×/0.30 and HC PL APO 20×/0.70 CS objective lens; Leica) in conjunction with LAS AF 2.2 software (Leica).

Statistical Analyses—Two-tailed Student’s t tests were used for two-group comparisons. Analysis of variance and appropriate post hoc analyses were used for comparisons of more than two groups. p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In an initial effort to screen genes associated with hippocampal neuronal development, we identified several genes, out of 9349 candidate genes, that exhibited significant changes in expression between E16 and adult hippocampus using the MouseWG-6 v2 Expression BeadChip (Illumina) kit (data not shown). We determined ADH5 to be an interesting candidate for regulating neuronal development due to its expression pattern and unrecognized role in neuronal development. Further examination of both mRNA and protein levels of ADH5 in the hippocampus revealed that its expression gradually declined from E16 to adulthood (Fig. 1A and B), which correlates with the development and maturation of hippocampal neurons (25, 26). We speculated that higher expression of ADH5 in E16 as compared with the postnatal stage may reflect an enrichment of low differentiated or undifferentiated neural stem or progenitor cells at early embryonic stage, which is gradually diluted following neuronal development. The decrease of ADH5 expression during neuronal development raises an intriguing question on whether ADH5 is involved in the regulation of neurite outgrowth, a requisite hallmark for neuron maturation. To this end, we first investigated whether altered ADH5 levels could impact neurite outgrowth of young hippocampal neurons (DIV2). As shown in Fig. 1C, overexpression of ADH5 in cultured mouse hippocampal neurons markedly repressed neurite outgrowth, whereas the catalytically inactive mutant of ADH5 (ADH5 (mt)) did not show any repressive effect. Conversely, knockdown of ADH5 by RNA interference in fetal hippocampal neurons significantly stimulated neurite projection (Fig. 1C). Also, the enhanced neurite extensions of ADH5−/− neurons, similar to ADH5 knockdown neurons, were inhibited by exogenous expression of ADH5 (Fig. 1D). To verify these observations in late neuronal development, hippocampal neurons were isolated from WT and ADH5 null mice, and their neurites were examined. ADH5−/− neurons exhibited much higher neurite complexity during in vitro culture (images were taken at DIV7; Fig. 1E). Finally, we examined the dendritic morphology of pyramidal neurons in hippocampal CA1 in the brain slices of WT and ADH5 null mice at postnatal day 16. Consistent with the observation in culture studies, loss of ADH5 not only increased the number of secondary dendrites and terminals in basal dendrites of pyramidal neurons, but also resulted in higher density in the dendritic branch in the region about 30–100 μm from the soma (Fig. 1, F–H). Neuronal differentiation also occurs in limited areas of adult brain, including the hippocampus and olfactory bulb, which designated adult neurogenesis. To identify the involvement of ADH5 in this specific neuronal differentiation, we further examined the effect of altered ADH5 activity on in vivo neurogenesis in the mouse SVZ-RMS-olfactory bulb (OB) system. As shown in Fig. 1I, injection of lentiviruses encoding WT ADH5, but not its catalytically inactivated mutant at SVZ, significantly repressed the newborn neuron numbers at OB, whereas no obvious change in dendritic morphologies of newborn neuron was observed. As
expected, injection of the ADH5 inhibitor C3 at SVZ region in adult mouse brain resulted in a significant increase in the number of newborn neurons in OB, indicating a role of endogenous ADH5 activity in negatively regulating SVZ-OB neurogenesis (Fig. 1J). Together, these data indicated that ADH5 functions as a negative regulator for both the development and the adult neuronal differentiation in mouse brain.

ADH5 functions as both a glutathione-dependent formaldehyde dehydrogenase and an S-nitrosoglutathione reductase (9, 27). Recent studies have established an important role of ADH5...
in denitrosating both S-nitrosogluthathione and specific S-nitro-
sated proteins (11, 28, 29). Considering that S-nitrosation of
HDAC2 is required for epigenetic activation of neuronal differ-
entiation-associated genes (7, 30), we hypothesize that ADH5
may antagonize neurogenesis by denitrosating HDAC2. To test
this, we first investigated whether S-nitrosated HDAC2 is a tar-
get of ADH5. Although HDAC2 was S-nitrosated in 293T cells
treated with NO donor S-nitrosocysteine, employment of an
ADH5 enzyme inhibitor (C3) or knock-out of endogenous
ADH5 elevated the level of S-nitrosated HDAC2 (Fig. 2, A and
B). These results demonstrated that endogenous ADH5 was
capable of denitrosating HDAC2 in cells. Furthermore, the lev-
els of S-nitrosated HDAC2 in adult hippocampus were signifi-
cantly higher than in E16 (Fig. 2C), which correlated with lower
protein level of ADH5 in adulthood (Fig. 1B). Subsequently, we
examined whether deconjugation of S-nitrosated HDAC2 is
involved in ADH5-mediated regulation of neurite growth.
A rescue experiment in mouse hippocampal neurons dem-on-
strated that ectopic overexpression of HDAC2(C262A/
C274A), an S-nitrosation-deficient HDAC2 mutant (7), signifi-
cantly inhibited neurite outgrowth induced by ADH5 silence
to an extent that is stronger than that exerted by wild-type
HDAC2 (Fig. 2D). These data raised a notion that deconju-
gation of S-nitrosated HDAC2 may be one of the factors contrib-
uting to ADH5-mediated repression of neurite growth.

We next investigated the role of ADH5 in the context of
directed differentiation of hNSCs toward neurons. hNSCs were
derived from human embryonic stem cells using our previous
approach (23), and they expressed the neural progenitor mark-
ers Nestin, Pax6, Sox2, and Ki67 (Fig. 3A) and could efficiently
differentiate into TuJ1-positive human neurons (Fig. 3, B and
F). Quantitative RT-PCR analysis demonstrated a dramatic
decrease in ADH5 mRNA levels in hNSC-derived neurons as
compared with their hNSC counterparts (~20-fold down-reg-
ulation, Fig. 3C), which is consistent with the observed down-
regulation of ADH5 during mouse neuronal development and
in vitro neuronal differentiation. To understand the conse-
quence of ADH5 down-regulation in human neuronal differen-
tiation, we overexpressed ADH5 in hNSCs by a lentiviral vec-
tor. ADH5 overexpression did not affect the expression of
hNSC markers including Sox2 and Nestin (Fig. 3D). However,
the migration ability of ADH5-overexpressed hNSCs was sub-
stantially compromised (Fig. 3E). Next, we initiated spontane-
ous neuronal differentiation from hNSCs. Immunofluores-
cence and qPCR analyses demonstrated that although empty
vector-transduced hNSCs were readily differentiated to MAP2,
TuJ1, GAD67, and synapsin-positive human neurons, ectopic
overexpression of ADH5 led to dramatic decrease of these
neuronal markers (Fig. 3, F–H). In addition, ADH5-over-
expressed cells exhibited clear non-neuronal morphology with
abnormally enlarged nuclei (Fig. 3F) and expressed high level of endo-
derm marker α-fetoprotein (AFP) (Fig. 3F). The repressive
effect of ADH5 on neuronal differentiation was completely
abrogated by inactivation of its catalytic sites or co-treatment
with ADH5 inhibitor (Fig. 3, F–I). Finally, we tested whether
treatment of hNSCs with an ADH5 inhibitor alone could facil-
itate their differentiation toward neurons. As demonstrated in
Fig. 3, J and K, a 6-day supplementation of the ADH5 inhibitor
C3 in hNSC differentiation medium significantly stimulated
the expression of neuronal markers MAP2 and GAD67. In sum-
mary, these results suggested that as in a mouse setting, ADH5
suppresses human neuronal differentiation in a catalytic activ-
ity-dependent manner.

Here we reveal a novel role for ADH5 as a negative regulator
of mammalian neuronal differentiation. To our knowledge,
this is the first study connecting a metabolic enzyme to the
regulation of human neurogenesis. Down-regulation of ADH5
may provide a permissive cellular environment allowing for
neuronal differentiation. Additionally, decreased expression of
ADH5 during neuronal differentiation may facilitate neurite
branching and projection. Together, our observations suggest
that inhibition of ADH5 may be utilized to facilitate adult neu-
rogenesis, especially in the contexts of aging and neurodegen-
erative diseases, but first, a better understanding of the mole-
cular mechanisms underlying ADH5 is needed. ADH5 neuronal
differentiation antagonism seems to depend on ADH5 catalytic
activity. It was reported that S-nitrosation of HDAC2 mediates
neurotrophin-dependent chromatin remodeling and the acti-
vation of neuronal development genes (7, 30), and likewise
HDAC inhibition is able to promote neuronal differentiation
(31). Our study reveals that ADH5 serves as a denitrosation
enzyme for HDAC2, and programme down-regulation of
AHD5 may lead to a derepression of HDAC2 by increasing its
S-nitrosation, which consequently facilitates specific gene
expression resulting in neuronal differentiation and develop-
ment. The possible involvement of other ADH5-catalyzed sub-
strates in the regulation of neuronal differentiation and devel-
opment requires further investigation.

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