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HISTONE DEACETYLASE 9 AS A NEGATIVE REGULATOR FOR CHOLINE ACETYLTRANSFERASE GENE IN NG108-15 NEURONAL CELLS

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Abstract—The biological function of histone deacetylases (HDACs), namely, repression of gene expression by removing an acetyl group from a histone N-terminal tail, plays an important role in numerous biological processes such as cell cycle, differentiation, and apoptosis in the development of individual tissues, including the brain. We previously showed the possible role of HDAC activity in the regulation of gene expression of choline acetyltransferase (ChAT), a specific marker for cholinergic neurons and their function, in NG108-15 neuronal cells as an *in vitro* model of cholinergic neurons. The objectives of the present study were to specify key HDACs and investigate the essential role of HDACs in ChAT gene regulation in NG108-15 cells. The experiments using different types of HDAC inhibitors indicated that class IIa HDACs substantially participate in the regulation of ChAT gene expression. In addition, HDAC9, a class IIa enzyme, was dramatically decreased at the protein levels, and dissociated from the promoter region of ChAT gene during neuronal differentiation. Furthermore, knockdown of HDAC9 by siRNA increased ChAT gene expression in undifferentiated cells. These findings demonstrate that HDAC9 is responsible for repressing ChAT gene expression in NG108-15 neuronal cells, and thus plays an important role in cholinergic differentiation.

Key words: histone deacetylase 9, histone deacetylase inhibitor, choline acetyltransferase, cholinergic neuron, NG108-15 cells.

In eukaryotes, genomic DNA is assembled with highly conserved histone proteins (H2A, H2B, H3, and H4) to configure the nucleosome, the fundamental unit of chromatin. The structure of chromatin is dynamically modulated by post-translational modification of the histone N-terminal tail, such as acetylation, methylation, phosphorylation, and ubiquitination (Kouzarides, 2007; Cairns, 2009). The pattern of covalent modification of histone tail, called histone code, influences transcriptional activity (Jenuwein and Allis, 2001; Khorasanizadeh, 2004); acetylation of N-terminal lysine residues, in particular, is a major source of chromatin remodeling. This covalent modification induces transcriptional activation by decreasing the affinity of N-terminal tails for DNA, resulting in relaxation of the nucleosome (Jenuwein and Allis, 2001; Hsieh and Gage, 2005). In contrast, histone deacetylases (HDACs; EC 3.5.1) remove the acetyl group from the lysine residues, resulting in a condensed chromatin structure, which prevents the access of transcription factors to their target elements, and thereby represses mRNA transcription (Ashraf and Ip, 1998; Wade, 2001; Hsieh and Gage, 2005). The steady state of histone acetylation is maintained by the balance between histone acetyltransferase (HAT) and HDAC activities. Recent progress in epigenetics has demonstrated that acetylation of N-terminal lysine residues of histone tail plays an essential role in numerous biological processes such as cell cycle, differentiation, and apoptosis in the development of individual tissues, including the brain.

In mammals, 18 HDACs have been identified and classified on the basis of their homology with their yeast counterparts. Class I enzymes (HDAC1, -2, -3, and -8), which are homologous to Rpd3 in yeast, exhibit nuclear localization and are ubiquitously expressed in most tissues (Yang and Seto, 2008). In contrast, class II HDACs, which are homologous to yeast Hda1, are selectively expressed in heart, skeletal muscle, and brain, and can generally translocate between the nucleus and cytoplasm in a phosphorylation-regulated manner (for review, see Verdin et al., 2003). In addition, these classes of enzyme have been further subdivided into classes IIa (HDAC4, -5, -7, and -9) and IIb (HDAC6 and -10). Class IIa enzymes are characterized by the presence of an N-terminal extension that interacts with positive and negative transcriptional cofactors, whereas the class IIb enzymes contain two catalytic domains (Verdin et al., 2003). Class III HDACs related to the yeast transcriptional repressor silent information regulator-2 (Sin2) and called sirtuins (SIRT1-7) are structurally and functionally distinct from all the other HDACs (Michishita et al., 2005). In addition, a new member of the HDAC family, HDAC11, has recently been identified and classified as a new class IV HDAC owing to its distinct structure (Gao et al., 2002). Class I, II, and IV HDACs share common features such as the dependence on zinc for their enzymatic activity, whereas class III HDACs are NAD-dependent (Haigis and Guarente, 2006). In embryonic mouse neural stem cells, trichostatin A (TSA), which inhib-

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Abbreviations: ChAT, choline acetyltransferase; ChIP, chromatin immunoprecipitation; CREB, cAMP-response element binding protein; dbcAMP, dibutyryl cyclic adenosine monophosphate; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDRP, histone deacetylase-related protein; MEF2, myocyte enhancer transcription factor 2; PBS, phosphate buffered saline; PKA, protein kinase A; SAHA, suberoylanilide hydroxamic acid; SEM, standard error of mean; TSA, trichostatin A; VPA, valproic acid.

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its all zinc-dependent HDAC activities (Yoshida et al., 1990; Carew et al., 2008), stimulated neuronal lineage progression, and morphological and electrophysiological maturation (Balasubramaniyan et al., 2006). Furthermore, valproic acid (VPA), which is a short-chain fatty acid widely used to treat epilepsy and bipolar mood disorder and has been defined as a direct inhibitor of class I and IIa HDAC activities (Phiel et al., 2001; Göttlicher et al., 2001; Bolden et al., 2006; Carew et al., 2008), promotes neuronal fate and inhibits glial fate through the induction of neurogenic transcription factors in adult rat neural stem cells (Hsieh et al., 2004). In addition, several lines of evidence indicate that HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and TSA are effective for treatment of several neurodegenerative disorders (Abel and Zukin, 2008). Thus, it is clear that HDACs play an important role in neuronal differentiation and functions. However, little information is available on the neurotransmitter phenotype specification. We previously showed that TSA induced mRNA expression of choline acetyltransferase (ChAT: acetyl-coenzyme A: choline O-acetyl transferase, ChAT; EC 2.3.1.6), which is widely recognized as a specific marker for cholinergic neurons and their functions, accompanied by an increased level of histone H3 and H4 acetylation, in undifferentiated NG108-15 cells as an *in vitro* model of cholinergic neurons (Aizawa and Yamamuro, 2010). This suggested that zinc-dependent HDACs are involved in the regulation of ChAT gene expression. The objectives of the present study were to specify key HDACs and investigate the essential role of HDACs in ChAT gene regulation in NG108-15 cells.

EXPERIMENTAL PROCEDURES

Materials

NG108-15 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), TRIzol reagent, Opti-MEM I reduced-serum medium, and Dynabeads M-280 sheep anti-rabbit IgG were obtained from Invitrogen Corporation (Carlsbad, CA, USA). The following HDAC inhibitors, trichostatin A from *Streptomyces* sp. (TSA), valproic acid sodium salt (VPA), and MC1568, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dibutyryl cyclic AMP (N^6, 2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt; dbcAMP) was also from Sigma-Aldrich. The rabbit polyclonal antibody against acetyl-histone H3 (ac-H3; $Lys^{9\ and\ 14}$) was purchased from Millipore Corporation (Lake Placid, NY, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was from Zymed Inc. (South San Francisco, CA, USA). HDAC family antibody set (rabbit polyclonal IgG) was obtained from BioVision (Mountain View, CA, USA). The rabbit polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The secondary antibody, horseradish peroxidase-linked donkey anti-rabbit IgG, was obtained from GE Healthcare (Waukesha, WI, USA).

Cell culture and treatments

NG108-15 cells were maintained in DMEM containing 10% FBS, 1% hypoxanthine-aminopterin-thymidine media supplement (Sigma), and a 1% antibiotic/antimycotic solution (Sigma) in a humidified incubator with 10% CO_2 in air at 37 °C. The medium was changed every 2–3 days. For HDAC inhibitor treatment, cells were seeded at a density of 5×10^5 cells/dish in 60-mm poly-L-lysine-coated cell culture dishes (Asahi Glass Co. Ltd., Tokyo, Japan) with DMEM containing 10% FBS at 24 h before treatments. After 24 h of plating, cells were exposed to TSA (100 nM), VPA (0.1, 0.5, and 1 mM), MC1568 (1, 5, and 10 μM), or vehicle (dimethyl sulfoxide; DMSO) for 48 h. The medium with HDAC inhibitors was replenished every 24 h. For immunofluorescence microscopy, cells were grown in the same conditions but on poly-L-lysine-coated coverslips (Asahi Glass Co. Ltd.).

For neuronal differentiation, sub-confluent cells were seeded at a density of 3×10^5 cells/dish as described previously. After 24 h of plating, cells were washed once with serum-free medium, and the medium was replaced with DMEM containing 1% FBS and 1 mM dbcAMP (dissolved in phosphate buffered saline; PBS) for 96 h. Morphological changes to the cells were monitored as an index of the differentiation.

Immunofluorescence microscopy

Cells cultured on coverslips were washed twice with PBS, fixed in 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS. Non-specific binding was eliminated by incubating the cells in blocking solution containing 1% bovine serum albumin for 30 min at room temperature. Then, cells were incubated with primary antibodies against ac-H3 (1:200 in blocking solution). Primary antibodies were detected by incubation with FITC-conjugated secondary antibody (1:400 in blocking solution) for an additional hour. The cells were mounted in the mounting medium supplemented with Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) to stain the nuclei and viewed using a fluorescence microscope (Olympus, Tokyo, Japan) with the same exposure time.

Total RNA extraction, reverse transcription and real-time PCR

Total RNA from cultured cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's directions and quantified spectrophotometrically after DNase I (Takara Bio Inc., Shiga, Japan) treatment. Total RNA (1 μg) of each sample was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI, USA) in a final volume of 20 μl, according to the manufacturer's instructions. For real-time PCR, reaction was performed using Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). PCR primers for real-time RT-PCR are listed in Table 1. Test samples were assayed in duplicate in 20-μl reaction mixtures containing 10 μl of reaction mix, 0.5 μM primers, 2 μl of cDNA, and 6 μl of nuclease-free H_2O. Non-template controls were also included. The thermal profile consisted of 2 min of denaturation at 95 °C, followed by 45 cycles of PCR at 95 °C for 5 s and 60 °C for 20 s. Following amplification, melting curve analysis was per-

Table 1. Primer sequences used in real-time RT-PCR

Target gene	Primer sequences	Product size (bp)
ChAT	5′-GCCAGTGGAAGAATCGTCAT-3′ 5′-TTGTGCATGTGAGTGTGTGG-3′	93
HDAC4	5′-GAGCTTGGCACACAGACTTG-3′ 5′-GTTGGGTAAGGATGGTGACG-3′	79
HDAC9	5′-GGCAGAATCCTCGGTCAGTA-3′ 5′-GCCTCATTTTCGGTCACATT-3′	90
GAPDH	5′-TGCCACCCAGAAGACTGTGG-3′ 5′-TTCAGCTCTGGGATGACCTT-3′	129