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Involvement of transcription factor XBP1s in the resistance of HDAC6 inhibitor Tubastatin A to superoxidation via acetylation-mediated proteasomal degradation

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ABSTRACT

HDAC6 is a major cytoplasmic deacetylase. XBP1s is a basic-region leucine zipper (bZIP) transcriptional factor. Despite their mutual involvement in the anti-oxidative process, there are no reports about their inter-protein interactions so far. Here we identified a direct link between HDAC6 inhibition and XBP1s transcription activity in anti-oxidative damage. We showed that the specific HDAC6 inhibitor Tubastatin A could up-regulate XBP1s transcriptional activity, thereby increasing anti-oxidative genes expression. Moreover, knock down of XBP1s could significantly abolish the cell growth protection afforded by Tubastatin A. We hypothesize that Tubastatin A acts to increase XBP1s protein levels that are dependent on its HDAC6 deacetylase inhibition via a mechanism involving acetylation-mediated proteasomal degradation, providing novel mechanistic insight into the anti-oxidative effects of HDAC6 inhibition.

1. Introduction

HDAC6 is the principal cytoplasmic deacetylase in mammalian cells [1]. HDAC6-specific substrates are varied, and include α-tubulin [2], cortactin [3], HSP90 [4], IFNαR [5], peroxiredoxin (Prx) I and Prx II [6]. Its involvement in deacetylation gives HDAC6 an important role in the progression of neurodegenerative diseases and lends itself to being a potential therapeutic target [6–8].

In addition to deacetylation, high levels of reactive oxygen damage cells are also believed to be associated with neurodegenerative disorders [9]. A potential interplay between deacetylation and oxidative stress can be found in data using Tubacin [6] and Tubastatin A [10], both highly selective HDAC6 inhibitors that have also showed good anti-oxidative activity.

Despite this prior work, the mechanism behind the anti-oxidative activity of HDAC6-specific inhibitors has still not been clarified. Two substrates have been found to be directly regulated by HDAC6: the cytoplasmic antioxidants enzymes peroxiredoxin (Prx) I and Prx II both appear to be involved in the anti-oxidative effects of HDAC6 inhibition [6]. Consistent with HDAC6 localization to the cytoplasm and its ability to deacetylate a range of cytoplasmic target proteins, it has been suggested that the effects of HDAC6 inhibition occur through a transcription-independent, local mechanism [6].

To this end, our work provide evidence that XBP1s, a bzip transcription factor that is involved in the mammalian unfolded protein response (UPR), could play an important role in the antioxidative activity of HDAC6 inhibition caused by Tubastatin A. This putative interaction between HDAC6 and nuclear transcription factor XBP1s provides evidence for a transcriptionally-regulated mechanism for HDAC6 function.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, New York, USA). Protein A-Agarose, anti-acetyl-tublin, and anti-β-actin antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-tubulin antibody was purchased from Epitomics (Burlingame, CA, USA) and Anti-acetyl-histone H3 antibody was from Millipore (Billerica, MA, USA). Anti-acetylated-lysine, anti-histone H3, anti-Flag and anti-HA antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Tubastatin A, SAHA and valproic acid (VPA) are gifts from Prof. Jingkang Shen (*Shanghai Institute of Materia Medica*). Tubacin was obtained from BioVision

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(Milpitas, CA, USA) and Niltubacin was from Enzo Life Sciences, Inc (Farmingdale, NY, USA).

2.2. Cell culture and cell proliferation assay

HEK293T and PC12 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, PR China). Cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 95% air and 5% carbon dioxide (CO_2) at 37 °C. Cell growth was assessed using an MTT assay as described previously [11].

2.3. RNA extraction and Q-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription and Q-PCR were conducted using the PrimeScript RT reagent and the SYBR Premix Ex TaqTM kits (Takara, China) at the ABI 7300 PCR system. Refer to Table 1 for relevant PCR primer sequences. All genes of interest were normalized to a GAPDH loading control and subsequent mRNA results were expressed as fold changes relative to GAPDH using the $2^{-\Delta\Delta CT}$ method [12].

2.4. Plasmids and transfection

The pFLAG-XBP1s-CMV2, pcDNA3-FLAG-HDAC6 and HA-Ubiquitin [13] were obtained from Addgene (Cambridge, MA, USA). XBP1s point mutants were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). Refer to Supplemental Table 1 for XBP1s point mutant primer sequences. The XBP1s siRNA sequence was synthesized by Shanghai GenePharma Co., Ltd. and was as fagca-3′ [14]. Ad-XBP1s was a gift from Dr. Yong Liu (The Institute for Nutritional Sciences (INS), Shanghai Institutes for Biological Sciences, CAS). Cell transfection was performed using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

2.5. WB (WB)

Cells were lysed, separated using SDS–PAGE, and were immunoblotted with the related primary antibodies. Resulting bands were detected using HRP-conjugated secondary antibodies and ECL reagent (GE Healthcare Life Science, Pittsburgh, PA, USA) according to the manufacturer's instructions. The quantification of band intensities was performed using *SmartView* software in conjunction with the FR-980A Gel Image Analysis System (Shanghai Furi Science and Technology Co., Ltd., Shanghai, PR China).

Table 1
Q-PCR primer sequences for the interested genes.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
PRDX5	GGCAGCATAGCCGGATCGGTG	CTCTGCCTGCCACGCACGTC
CAT	CCAGACACTCACCGCCACCG	TGGTAGTTGGCCACGCGAGC
PDIA5	AAGGCTGCCACCCAAGTGCG	GCAGATGGTGGGGTAGCCACG
SOD1	TTCGTTTCCTGCGGCGGCTT	GGACCGTCGCCCTTCAGCAC
TRX-1	GGCCGCTGCGGGAGACAAG	AGGTCCACACCACGTGGCAGA
NQO-1	GCCGCCTGAGCCCGGATATTG	AATGGCGGGCACCCCAAACC
ERO1a	TGGGGCTTGCTCGTCGGACT	CAGAAACACCGCTGTGCCGC
EDEM1	TGGCTTCATCGGCCAAGGCG	CCACCGCCGCCCAAAGCATA
SEC61a	TGGGTCCGCCACACCTCAGT	CCTGTGTCGGGGCAAGTGGC
GAPDH	GGGGCTCTCTGCTCCTCCCTG	CCAGGCGTCCGATACGGCCA
XBP1s	GAGTCCGCAGCAGGTGCAGG	GGGTCCAACTTGTCCAGAATGCCC
XBP1u	GAGTCCGCAGCACTCAGACTACG	GGGTCCAACTTGTCCAGAATGCCC

2.6. Immunoprecipitation for the acetylated XBP1s detection and acetylation site identification

HEK-293T cells that were overexpressed Flag-XBP1s were lysed in RIPA buffer. Cell extracts were then immunoprecipitated with Protein A-Agarose beads conjugated to the anti-Flag antibody. The precipitated complexes were then analyzed through WB with either an anti-acetylated-Lysine or an anti-Flag antibody.

Acetylation site identification was performed by separating the previously precipitated IP sample through SDS–PAGE. A band of approximately~55 kDa was recovered and completely digested with trypsin. Spectrometric identification was performed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis (MALDI-TOF MS) and the final peptide mapping analysis was performed using the Mascot tool.

2.7. Co-immunoprecipitation to detect XBP1s ubiquitination state

For the co-immunoprecipitation of Flag-XBP1s and HA-Ubiquitin, HEK293T cells were co-transfected with Flag-XBP1s and HA-Ubiquitin plasmids. Cell lysates were then immunoprecipitated using Protein A-Agarose beads conjugated to the anti-Flag antibody. Precipitated complexes were then immunoblotted with both the anti-HA and anti-Flag antibodies.

2.8. Statistical analyses

Data are presented as means with standard deviation (±SD). Statistical analyses between groups were performed using two-tailed Student's *t* test with GraphPad software. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Tubastatin A up-regulates anti-oxidative gene expression related to transcription factor XBP1s

Hydrogen peroxide (H_2O_2) was used to induce oxidative stress and neuronal damage. Tubastatin A, an HDAC6 inhibitor known to have neuroprotective effects [10], was used to inhibit HDAC6 activity. Prior work has elucidated genes important to human anti-oxidant responses, which include peroxidases, superoxide dismutases and thiol redox regulating genes [15]. Based on this prior characterization, peroxidase genes *PRX5* and *CAT*, superoxide dismutase gene *SOD*, thiol redox regulating genes *TRX-1* and *PDIA5*, and Nrf2 target gene *NQO-1* were selected to investigate the hypothesis that HDAC6 inhibition affects the transcription level of anti-oxidative genes.

First, Tubastatin A was found to significantly reverse H_2O_2-induced inhibition of PC12 cell growth (Fig. 1A). This reversal was evident at both the 24 and 48 h marks, indicating that Tubastatin A could protect PC12 cells from H_2O_2-induced oxidative damage. Six anti-oxidative stress-related genes were detected with Q-PCR in cells treated with Tubastatin A and H_2O_2. Tubastatin A treatment did not affect the expression of *SOD*, *TRX1* and *NQO-1*, but significantly increased the expression of *CAT*, *PRDX5* and *PDIA5* genes. This increase was significant, particularly after H_2O_2 treatment (Fig. 1B). These findings suggest that HDAC6 inhibition could regulate some anti-oxidative genes expression.

All three up-regulated genes, *CAT*, *PRDX5* and *PDIA5*, have been reported to be regulated by transcription factor X-box binding protein-1 (XBP1s) [16]. This finding prompted us to speculate that the transcriptional activity of XBP1s had also increased. Previous research on XBP1s has shown that it regulates ERAD genes such as *EDEM1*, *SEC61α* and *ERO1α*. All three of these genes were

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