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Histone deacetylase inhibitor SAHA induces ERα degradation in breast cancer MCF-7 cells by CHIP-mediated ubiquitin pathway and inhibits survival signaling

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ABSTRACT

Estrogen receptor α (ERα) plays an important role in the development and progression of breast cancer, and recent studies showed that ERα expression is associated with resistance to hormonal therapy. Therefore, a number of studies have explored ways to deplete ERα from breast cancer cells as a new therapy especially for hormone-refractory breast cancer. We reported here that suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, effectively depletes ERα in breast cancer MCF-7 cells. However, the intrinsic mechanisms by which SAHA decreases ERα levels are not clear. Our present data demonstrated that both inhibition of ERα mRNA level and promotion of ERα degradation by the proteasome contribute to SAHA-induced ERα depletion, indicating that SAHA may exert its effects through transcriptional and posttranslational mechanisms. Furthermore, the decrease of ERα protein level in MCF-7 cells after SAHA treatment is mainly the result of its rapid degradation by the ubiquitin-proteasome pathway rather than transcriptional inhibition. In addition, we showed that inactivation of the heat shock protein-90 (Hsp90) is involved in SAHA-induced ERα degradation, and ubiquitin ligase CHIP (C-terminal Hsc70 interacting protein) enhances SAHA-induced ERα degradation. SAHA-induced ERα depletion is paralleled with reduction of transcriptional activity of ERα and SAHA is able to effectively inhibit cell proliferation and induce apoptosis of MCF-7 cells. Taken together, our results revealed a mechanism for SAHA-induced ERα degradation and indicated that SAHA is a suitable pharmacological agent for depletion of ERα and a potential choice for breast cancer expressing high ERα.

1. Introduction

Breast cancer is the most common form of malignant disease in woman worldwide. It has been reported that ERα plays a critical role in the initiation and progression of breast cancer because approximately 70% of primary breast cancers are ERα positive [1,2]. As a result, ERα has become an important target in the treatment of hormone-responsive breast cancer. Unfortunately, most patients initially responding to anti-estrogen therapies, such as tamoxifen, which is a standard component of front-line therapy for ERα positive breast cancer, will eventually become resistant to those therapies [3]. Aromatase inhibitors (AIs) are new drugs used for endocrine treatment of post-menopausal breast cancer and have demonstrated efficacy in patients with breast cancer resistant to anti-estrogens. However, resistance to AIs has also been observed. The potential mechanisms of endocrine resistance are not fully understood, but evidence suggests

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that tamoxifen-resistant tumors regrowth may be associated with ERα signaling. Moreover, complex interactions between ERα and growth factor signaling pathways are also involved, which is thought to be one of the determinants of endocrine resistance [4–6]. Therefore, depletion of ERα from breast cancer cells may be a particularly powerful approach to block ERα signaling, especially ERα/growth factor crosstalk, preventing the development of endocrine resistance ultimately.

Unliganded ERα, like other steroid hormone receptors, is maintained in a ligand-binding competent conformation by associating with various Hsp90-based chaperone complexes [7]. Hsp90 inhibitors, such as geldanamycin (GA), can inhibit Hsp90 molecular chaperone function and induce ERα degradation through the ubiquitin-proteasome pathway [8,9]. Recently, it was found that carboxyl terminus of Hsc70-interacting protein (CHIP), which is a co-chaperone of Hsp90/Hsp70 complex, is involved in GA-induced ERα degradation as an ubiquitin ligase [10]. However, anti-estrogen fulvestrant, which also induces ERα degradation by dissociating the complex of Hsp90 with ERα, reducing the interaction between CHIP and ERα after treatment with GA, indicating that distinct downstream pathways exist for ERα degradation by treatment with different drugs [10]. Therefore, it is necessary for us to elucidate the molecular mechanism of new drug-induced-ERα degradation.

Histone deacetylase (HDAC) inhibitors, a promising class of antitumor agents, can block the proliferation and induce cell death in a wide variety of transformed cells [11]. However, the antitumor mechanism of HDAC inhibitors has not been completely elucidated. It has been proved that HDAC inhibitors selectively affect gene transcription by acetylation of histones, such as SAHA and LAQ824, which transcriptionally up-regulate p21 and increase p27 expression. This is associated with cell cycle arrest and apoptosis in cancer cells, and is shown to induce in vivo regression of tumors [12–14]. HDAC inhibitors-induced mitotic defect by causing aberrant acetylation of histones in heterochromatin and centromere domains per se can result in cell death by either apoptosis or mitotic death/catastrophe [15]. It has also been shown that SAHA can induce polyploidy and lead to cell senescence in transformed cells [16]. These findings indicated that HDAC inhibitors can cause tumor cell growth arrest, apoptosis, mitotic cell death and polyploidy to exert antitumor effects.

Recently, a number of studies showed that the antitumor effects of HDAC inhibitors may be associated with Hsp90 acetylation induced by HDAC inhibitors, such as depsipeptide (FK228), LAQ824 and SAHA. It has been proposed that Hsp90 acetylation correlates with inactivation of Hsp90 and induces Hsp90 client proteins disassociation from Hsp90 molecular complex, e.g. Raf-1, androgen receptor (AR) and HER2 [17–19].

An earlier research suggested that HDAC inhibitors deplete ERα protein [20]. However, little is known regarding the molecular mechanism of ERα depletion by HDAC inhibitors. Suberoylanilide hydroxamic acid (SAHA) is an HDAC inhibitor, which is in phase I/II clinical trials and has shown antitumor activity in hematologic and solid tumors at doses well tolerated by patients [21,22]. In present studies, we showed that SAHA can suppress ERα mRNA level and induce ERα degradation in breast cancer MCF-7 cells. The molecular mechanism of SAHA-induced ERα degradation is associated with inactivation of Hsp90, and co-chaperone CHIP of Hsp90 is also involved as E3 ubiquitin ligase. The effect of SAHA on ERα degradation is augmented by CHIP overexpression, but not CHIP mutants' overexpression, suggesting that CHIP participates in SAHA-induced ERα degradation. Most importantly, SAHA-induced ERα depletion results in down-regulation of ERα transcriptional activity and inhibition of cells proliferation in MCF-7 cells. Therefore, SAHA may exert its antitumor effect via depletion of ERα and inactivation of the crosstalk between ERα and growth receptors signaling pathways.

2. Materials and methods

2.1. Cell, reagents and plasmids

The breast cancer cell line, MCF-7 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Suberoylanilide hydroxamic acid (SAHA) was offered by AstraZeneca Company (Macclesfield, SK, UK). 17β-Estradiol, 4-hydroxytamoxifen, and MG-132 were purchased from Sigma Chemical Company (St. Louis, MO, USA). ICI 182,780 was purchased from Tocris Cookson Ltd. (Ellisville, MO, USA). Antibodies for Raf-1, phospho-AKT, AKT, phospho-ERK, ERK, acetylated-lysine, survivin, Myc, actin and ubiquitin were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-ERα and anti-CDK4 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Hsp90 antibody and anti-Hsp70 antibody were obtained from Stressgen Biotechnologies Corporation (Victoria, BC, Canada). Anti-CHIP antibody was purchased from Abcam Ltd. (Cambridge, UK). Lipofectamine 2000 was obtained from Invitrogen Corporation (Rockville, MD, USA). The Myc-CHIP (WT), Myc-CHIP (ΔTPR), and Myc-CHIP (ΔU-box) constructs were kindly provided by Dr. Yili Yang (NIH/NCI). ERE-pS2-Luc was kindly offered by Dr. Qi-Nong Ye (Beijing Institute of Biotechnology, Beijing, China).

2.2. Cell culture and transient transfection

MCF-7 cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in 5% CO_2. Before experiments, cells were cultured in hormone-free medium (phenol red-free MEM with 5% charcoal-stripped FBS) for 3 days. For transient transfection, cells were transfected with an equal amount of total plasmid DNA by using Lipofectamine 2000 according to the manufacturer's guidelines.

2.3. Stable transfection

MCF-7 cells were transfected with pcDNA3.1-Myc-CHIP (WT), pcDNA3.1-Myc-CHIP (ΔTPR), pcDNA3.1-Myc-CHIP (ΔU-box), or empty vector by using Lipofectamine 2000 and selected in growth medium containing 800 μg/ml G418 for 3 weeks. Then drug-resistant colonies were chosen and expanded in growth medium containing 300 μg/ml G418. The expression of Myc-CHIP (WT), Myc-CHIP (ΔTPR), and Myc-CHIP (ΔU-box) in stable cell lines (MCF-7) were detected by Western blot with anti-Myc antibody. In parallel, several empty plasmid-transfected clones were randomly selected and used as control cells.

2.4. Western blot, immunoprecipitation analysis and luciferase assay

For Western blot analysis, control or SAHA-treated MCF-7 cells were lysed in Laemmli buffer (Bio-Rad Laboratories, CA, USA), approximately 60 μg of total proteins were resolved on SDS polyacrylamide gels and immunoblot was performed as previously described [19]. For immunoprecipitation experiments, cellular extracts from approximately 1×10^7 cells were prepared in RIPA buffer, approximately 400 μg of total proteins were used for immunoprecipitation analysis followed by the procedure previously described [19]. For luciferase assay, cell lysates were prepared and carried out as recommended by Luciferase Assay System (Promega). Data were expressed as mean ± S.E.M. Statistical analysis was performed by Student's *t*-test and *P* values less than 0.05 were considered significant.

2.5. RT-PCR

Total RNA was extracted from cultured cells with TRIzol reagent according to the manufactured guidelines (Invitrogen). Reverse transcription (RT) was performed using First Strand cDNA Synthesis Kit (Fermentas). Primer pairs used for ERα PCR were as follows: forward, 5′-TGATCCTACCAGACCCTTCA-3′, and reverse, 5′-TCCTGTCCAAGAGCAAGTT-3′. G3PDH was amplified and used as a standard for the PCR reaction.

2.6. Cell proliferation assay

MCF-7 cells were plated into 96-well plates at a density of 6×10^4/well and cultured in hormone-free medium. After 3 days, the medium was replaced with fresh medium in the presence or absence of 1.25–10 μM SAHA. At appropriate time points, the percentages of viable cells after treatment were measured using the MTT assay. Each experiment was performed three times.

3. Results

3.1. SAHA depletes ERα via the ubiquitin-proteasome pathway in MCF-7 cells

To investigate the effect of SAHA on ERα expression, we used SAHA to treat breast cancer MCF-7 cells, which express high ERα protein, the results showed that SAHA induces dose- and time-dependent inhibition of ERα protein level (Fig. 1A). Similar inhibition was observed with depsipeptide (FK228) and trichostatin A (data not shown), which are type I and pan-histone deacetylase inhibitors, respectively [20]. Previous reports showed that HDAC inhibitors like LAQ824 exert their effects on depleting androgen receptor through transcriptional and posttranslational mechanisms [18]. To determine whether SAHA-mediated ERα depletion is due to the same mechanisms, we treated MCF-7 cells with SAHA for 6–24 h and

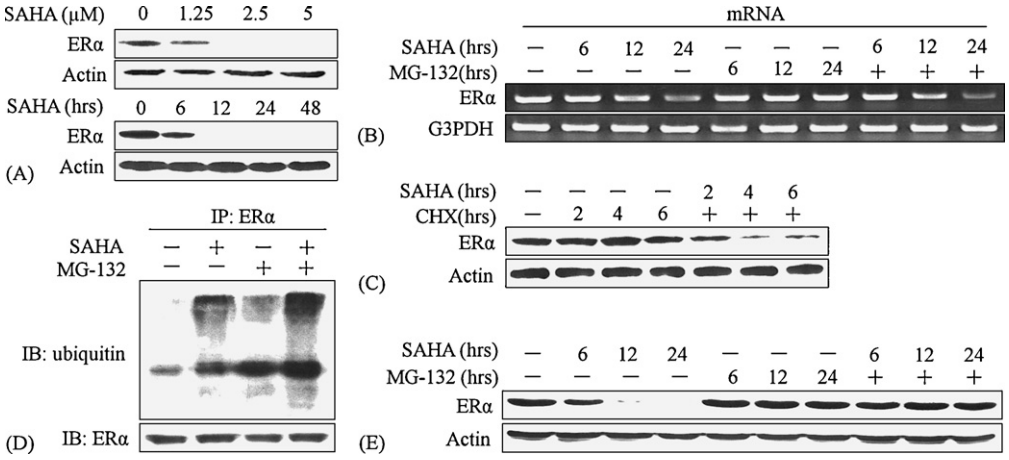

Fig. 1 – SAHA induces ERα degradation via the ubiquitin-proteasome pathway in MCF-7 cells. (A) MCF-7 cells were treated with different concentrations of SAHA for 24 h or treated with 5 μM SAHA at indicated times. Cell lysate was prepared and the protein level of ERα was examined with anti-ERα antibody by Western blot. (B) MCF-7 cells were treated with SAHA at indicated times in the presence of proteasome inhibitor MG-132 or in the absence of MG-132 (5 μM). Total RNA was extracted and mRNA level of ERα was detected by RT-PCR analysis, G3PDH served as loading control. (C) MCF-7 cells were treated with an inhibitor of protein synthesis, 100 μM cycloheximide (CHX) for 30 min, followed by addition of SAHA (5 μM) at indicated times. As a control, MCF-7 cells were treated with 100 μM cycloheximide (CHX) for 2, 4 and 6 h alone. The protein level of ERα was detected by Western blot with anti-ERα antibody. (D) MCF-7 cells were treated with SAHA (5 μM) for 4 h in the presence of proteasome inhibitor MG-132 or in the absence of MG-132 (5 μM). MG-132 was added to the cells 30 min before and continued during the SAHA treatment. Cell lysate was lysed in RIPA buffer and immunoprecipitated (IP) with anti-ERα antibody, resolved by SDS-PAGE, and immunoblotted (IB) with anti-ubiquitin antibody. (E) MCF-7 cells were treated with SAHA (5 μM) at indicated times in the presence of proteasome inhibitor MG-132 or in the absence of MG-132 (5 μM). As a control, MCF-7 cells were treated with MG-132 (5 μM) for 2, 4 and 6 h alone. The level of ERα protein was detected by Western blot with anti-ERα antibody.

performed RT-PCR analysis to detect ERα mRNA level. Comparison of the declining speed of ERα in protein and in mRNA levels showed that, the remarkable decline of ERα protein appeared at 6 h followed by the SAHA exposure, whereas the faint decline of ERα in mRNA level appeared until SAHA treatment for 12 h (Fig. 1B). The lagging of transcriptional inhibition suggested that SAHA may decline ERα protein level mainly via affecting its stability. To prove this hypothesis, we treated MCF-7 cells for various times with SAHA, in the presence or absence of cyclohexamide (CHX), an inhibitor of protein synthesis, in the presence or absence of SAHA, and then measured the relative ERα protein level in MCF-7 cells. As shown in Fig. 1C, ERα protein level decreases faster in the cells treated with SAHA in the presence of CHX than in the cells treated with CHX alone. This result supported that inhibitory effects of SAHA on ERα are mainly due to accelerate ERα degradation in MCF-7 cells. To determine whether the ubiquitin-proteasome system is responsible for SAHA-induced ERα degradation, we treated MCF-7 cells with SAHA at indicated times in the presence or absence of proteasome inhibitor MG-132. ERα protein was immunoprecipitated and its ubiquitination status was examined by Western blot with anti-ubiquitin antibody. Fig. 1D shows that SAHA treatment induces ubiquitination of ERα and this effect is evidently enhanced by cotreatment of SAHA and MG-132. Furthermore, the proteasome inhibitor MG-132 prevents SAHA-induced ERα degradation (Fig. 1E), suggesting that SAHA could induce ERα degradation via the ubiquitin-proteasome pathway. Although proteasome inhibitor MG-132 can completely prevent SAHA-induced ERα protein degradation, it has no effect on SAHA-induced decline on ERα mRNA level (Fig. 1B).

3.2. *SAHA induces Hsp90 acetylation and Hsp90-ERα complex disassociation in MCF-7 cells*

It has been discovered that the association of ERα with the heat shock protein-90 (Hsp90) molecular chaperone complex is required for its stability and function [7]. Recently, it was shown that HDAC inhibitors induce acetylation of Hsp90, which inhibits its ATP binding and chaperone association with its client proteins [17–19]. Consistent with these studies, we next determined whether Hsp90 acetylation is involved in SAHA-induced ERα depletion. Further experiments were then performed to assess the effect of SAHA on acetylation of Hsp90 and ERα by immunoprecipitation with anti-acetylated lysine antibody and immunoblotting with anti-Hsp90 or anti-ERα antibodies, respectively. The results showed that treatment with 5 μM SAHA as short as 2 h has already induced acetylation of Hsp90 in MCF-7 cells, and the effect was enhanced at 4 h but without significantly affecting the protein

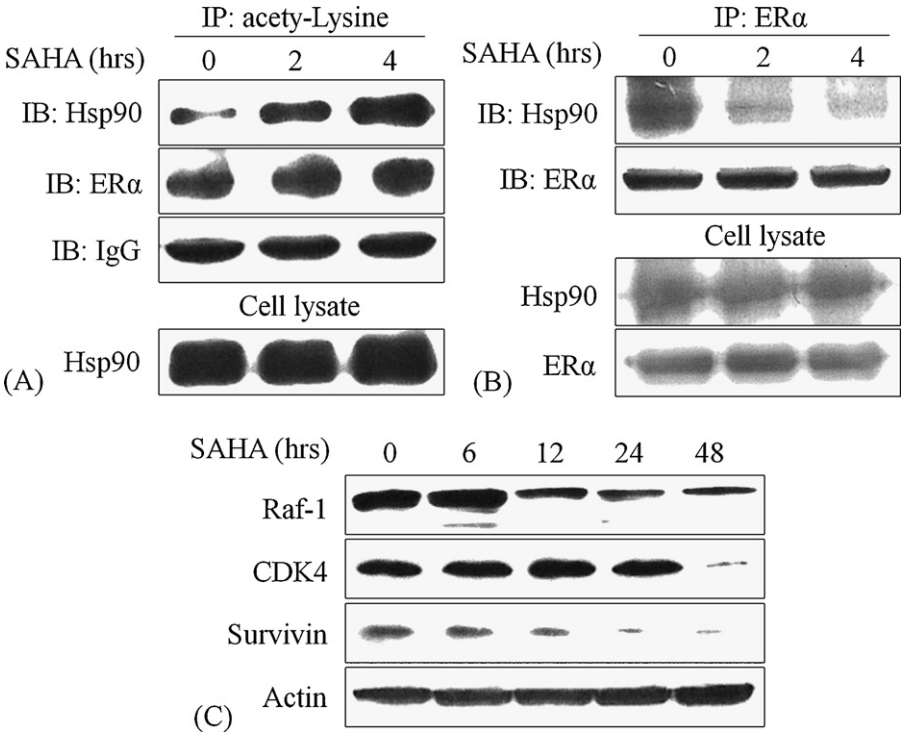

Fig. 2 – SAHA acetylates Hsp90 and induces dissociation of ERα from Hsp90 chaperone complex and depletes Hsp90 client proteins in MCF-7 cells. (A) SAHA induces Hsp90 acetylation. MCF-7 cells, treated with SAHA (5 μM) at indicated times, were lysed in RIPA buffer. Acetylated lysine was immunoprecipitated (IP) with anti-acetylated lysine antibody, resolved by SDS-PAGE, and immunoblotted (IB) with anti-Hsp90 or anti-ERα antibody. The level of Hsp90 in cell lysate was used as the loading control. (B) SAHA disassociates the binding between ERα and Hsp90. MCF-7 cells were treated with SAHA (5 μM) at indicated times, cell lysate was lysed in RIPA buffer and immunoprecipitated (IP) using anti-ERα antibody, resolved by SDS-PAGE, and immunoblotted (IB) with anti-Hsp90 or anti-ERα antibody. The levels of Hsp90 and ERα in cell lysate were used as the loading control. (C) SAHA depletes Hsp90 client proteins. Western blot analyses of Raf-1, CDK4, survivin and actin in the cell lysate from MCF-7 cells treated with SAHA (5 μM) for 6–48 h.

level of Hsp90 in MCF-7 cells. Interestingly, in the same conditions acetylation of ERα is not observed (Fig. 2A). Next, we estimated the effect of SAHA on the association of Hsp90-ERα. Following treatment with SAHA for 2 or 4 h, Hsp90-ERα coimmunoprecipitation assay was done. As shown in Fig. 2B, SAHA induces the disassociation of Hsp90 and ERα paralleled by increased acetylation of Hsp90. These data implied that SAHA induces Hsp90 acetylation and Hsp90-ERα disassociation in MCF-7 cells, resulting in ERα degradation via the ubiquitin-proteasome pathway. Previous studies showed that inactivation of Hsp90 molecular chaperone could induce depletion of its client proteins such as AKT, Raf-1, and steroid receptors [23–25]. We further evaluated the levels of Hsp90 client proteins such as Raf-1, CDK4 and survivin. Fig. 2C shows that SAHA also mediates the depletion of these Hsp90 clients. These results further indicated that SAHA-induced Hsp90 acetylation leads to disruption of its chaperone function and decrease of its client proteins.

3.3. *CHIP participates in SAHA-induced ERα degradation as an ubiquitin ligase*

Previous studies showed that carboxyl-terminus of HSC70-interecting protein (CHIP) is a U-box-containing E3 ubiquitin ligase that binds through its tetratricopeptide repeat (TPR) domain to independent TPR acceptor sites on Hsp90 and Hsp70 [26,27]. CHIP has been shown to facilitate the ubiquitination of Hsp90 client proteins, such as p53 and ErbB2 [28,29]. Most recently, CHIP has been reported to play a role in GA-induced degradation of ERα [10]. Whether CHIP also plays a role in SAHA-induced ERα degradation has not been investigated. To determine whether CHIP participates in SAHA-induced ERα degradation, we transfected wild type CHIP and deficient mutant plasmids (ΔTPR and ΔU-box) into MCF-7 cells and established the stable transfected cell lines. As a control, MCF-7-Mock (MCF-7 cells stably transfected with empty vector) was also established. We treated MCF-7-Mock cells and MCF-7-CHIP (WT) with SAHA at indicated times. Fig. 3A shows that ERα degradation is evidently accelerated in MCF-7-CHIP (WT) cells compared with MCF-7-Mock cells. It has been reported that CHIP associates with ERα and promotes ERα ubiquitination in MCF-7 cells, we wonder to investigate the function of CHIP in the case of SAHA-induced ERα degradation. We treated MCF-7-CHIP (WT) cells with SAHA for 2 or 4 h, ERα protein was immunoprecipitated with anti-ERα antibody, and coimmunoprecipitated CHIP was detected by immunoblotting with anti-Myc antibody. As shown in Fig. 3B, SAHA enhances association of CHIP with ERα in a time-dependent manner. We next examined whether CHIP could act as an ubiquitin ligase mediating SAHA-induced ERα degradation. MCF-7-Mock cells and MCF-7-CHIP (WT) cells were treated with SAHA for 2 h, respectively, ubiquitination of ERα was slightly increased in MCF-7-CHIP (WT) cells treated with SAHA (Fig. 3C), suggesting a role for CHIP in SAHA-induced ERα ubiquitination. Therefore, these results suggested that CHIP, by facilitating ERα ubiquitination, targets ERα for proteasome-mediated degradation. Furthermore, we evaluated whether overexpression of the deletion mutant CHIP including CHIP (ΔTPR) and CHIP (ΔU-box) could exert E3 ubiquitin ligase function in SAHA-induced ERα degradation. As shown in Fig. 3D, overexpression of CHIP (ΔU-box) and CHIP (ΔTPR) mutant, but not CHIP (WT), have no effects on SAHA-induced ERα degradation. These data indicated that intact CHIP is necessary to mediate SAHA-induced ERα degradation.

3.4. *SAHA down-regulates ERα transcriptional activity and blocks survival signaling in MCF-7 cells*

We have confirmed that SAHA is capable to depleting ERα expression through two mechanisms involving inhibition of ERα mRNA level and induction of ERα degradation via CHIP-mediated ubiquitin-proteasome pathway. To determine whether SAHA-induced ERα depletion is associated with attenuated ERα transcriptional activity, following treatment with SAHA for 6 h, we transiently transfected MCF-7 cells with an ERE-pS2-Luc reporter plasmid, then stimulated with E_2 for 24 h and analyzed with luciferase assay. As shown in Fig. 4A, SAHA decreases E2-induced ERα transcriptional activity in a dose dependent manner accompanied with remarkably decline of ERα protein level at same time. We assume that the inhibition effect of SAHA on ERα transcriptional activity is indirectly caused by ERα protein degradation, more experiments using stable transfected cell lines, MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box) further demonstrated that SAHA causes a stronger decrease of ERα transcriptional activity inMCF-7-CHIP (WT) cells but not in MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box) cells, which is consistent with its effect on depleting ERα from different cell lines (Fig. 3D).

We further evaluated the effects of SAHA on survival signaling pathways in MCF-7 cells. As shown in Fig. 4B, treatment with SAHA resulted in the reduction of phosphorylated ERK or phosphorylated AKT in a time-dependent manner, indicating that ERK and AKT activities are inhibited in these cells. Coincidence with the inhibition of ERK and AKT activities, the level of cleaved-PARP was increased, suggesting that inhibition of the Raf-1/MEK/ERK and PI3K/AKT survival signaling pathways is involved in the SAHA-induced apoptosis. Next, MCF-7 stable transfected cells were treated with SAHA for 24 h; the result showed that CHIP overexpression enhances the effect of SAHA on inactivating survival signaling pathways (Fig. 4B).

We have showed that treatment with 5 μM SAHA is able to induce ERα degradation and inhibit ERα transcriptional activity. To examine the growth inhibitory effect of SAHA in MCF-7 cells, MCF-7 cells were treated with SAHA for 0–72 h. MTT assay demonstrated that SAHA causes dose and time-dependent decrease in cell viability. The viability of MCF-7 cells was inhibited by 60% after 72 h, suggesting that SAHA is effective in inhibiting MCF-7 cell proliferation (Fig. 4C). Furthermore, to evaluate the correlation between SAHA-induced cytotoxicity and ERα depletion in MCF-7 cells, we compared the growth inhibitory effect of SAHA on MCF-7 cells with that on MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7 (ΔU-box) cells. The result showed that the cytotoxicity of SAHA is obviously enhanced in MCF-7-CHIP (WT) cells, whereas no difference among MCF-7 cells, MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box) cells. It is indicated that promoting ERα depletion by CHIP overexpression could enhance SAHA cytotoxicity (Fig. 4C). Taken together, SAHA down-regulates

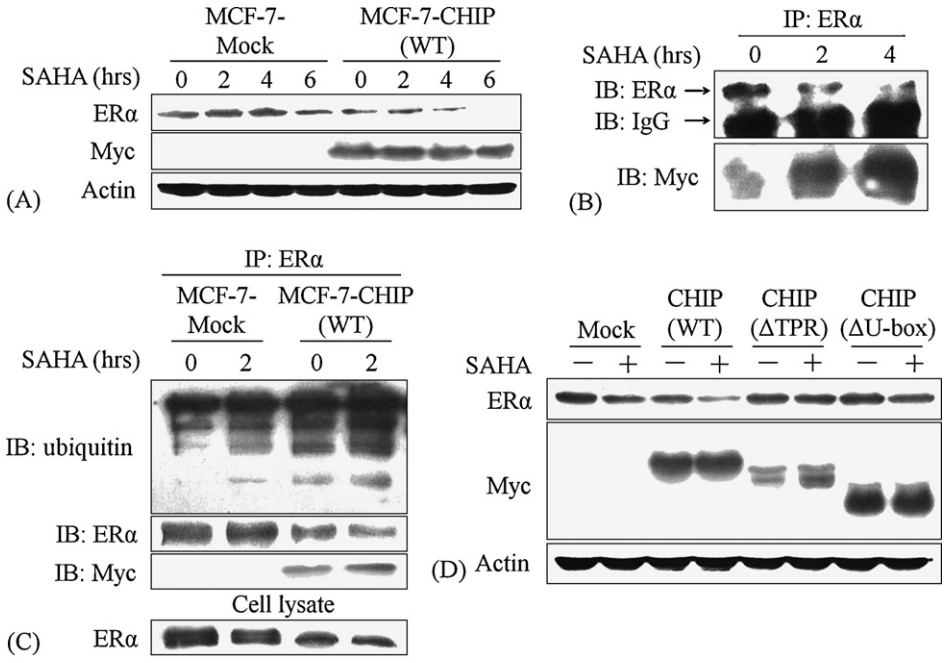

Fig. 3 – CHIP is required for SAHA-induced ERα degradation in MCF-7 cells. (A) Overexpression of CHIP promotes SAHA-induced ERα degradation. MCF-7 cells or MCF-7-CHIP (WT) cells were treated with SAHA (5 μM) at indicated times, cell lysate was prepared and the protein levels of ERα and CHIP were detected by Western blot with anti-ERα and anti-Myc antibody, respectively. (B) SAHA enhances CHIP-ERα interaction. MCF-7-CHIP (WT) cells were treated with SAHA (5 μM) at indicated times, and cell lysate was lysed in RIPA buffer and immunoprecipitated (IP) using anti-ERα antibody, resolved by SDS-PAGE, and immunoblotted (IB) with anti-ERα or anti-Myc antibody. (C) CHIP enhances SAHA-induced ERα ubiquitination. Immunoprecipitation (IP) was performed with anti-ERα antibody and immunoblotted (IB) with anti-ubiquitin, anti-ERα and anti-Myc antibody, respectively in the RIPA buffer from MCF-7 or MCF-7-CHIP (WT) cells treated with SAHA (5 μM) for 2 h. The level of ERα in cell lysate was used as the loading control. (D) CHIP's function is dependent on both TPR and U-box domain. SAHA (5 μM) treated these stable transfected cells (MCF-7-Mock, MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box)) for 6 h. The expression of exogenesis CHIP and its mutants were detected by anti-Myc antibody. ERα protein was detected by Western blot with anti-ERα.

ERα transcriptional activity and blocks survival signaling in MCF-7 cells via CHIP-mediated mechanism.

4. Discussion

It has been proposed that levels of ERα are commonly increased in premalignant and malignant breast lesions, and ERα plays a crucial role for the growth and survival of breast cancer cells. Recently, accumulated evidences also have shown that complex interactions between ERα and growth factor signaling pathways are one of the potential mechanisms of endocrine resistance to breast cancer [4–6]. Thus, ERα becomes an important target for the treatment of breast cancer. Depletion of ERα protein by Hsp90 inhibitors or ERα-small interfering RNA (siRNA) has been shown to suppress breast tumor growth in vivo [8,9,30]. Therefore, depletion of ERα from breast cancer cells is a rational therapeutic strategy not only for primary estrogen-dependent breast cancer but also for hormone-refractory breast cancer. Our experiments confirmed that SAHA, a HDAC inhibitor, currently in human clinical trials, is able to deplete ERα in breast cancer MCF-7 cells.

Our data showed that SAHA depletes ERα through two mechanisms: attenuation of its mRNA level and promotion of its degradation by ubiquitin-proteasome pathway. It is similar to what has been previously observed that HDAC inhibitor LAQ824 and TSA deplete HER2, Bcr-Abl, and AR through transcriptional and posttranslational mechanisms. It seems that HDAC inhibitors-induced degradation of oncoproteins maybe commonly accompanied by down-regulation of their mRNA levels. However, recent report showed that TSA has no effect on the levels of HIF-1α mRNA transcripts while it induces rapid degradation of HIF-1α protein [31], suggesting that the effect of HDAC inhibitors is different for various types of Hsp90 client proteins. Our current study has focused on the molecular mechanism of SAHA-induced ERα degradation. Previous report showed that TSA, another HDAC inhibitor, induced proteasome-independent down-regulation of ERα [32]. However, in this study, we observed that MG-132, a proteasome inhibitor, could effectively inhibit SAHA-induced ERα degradation and enhance ERα ubiquitination. Therefore, our data indicated that SAHA induces ERα degradation via the ubiquitin-proteasome pathway. The basis for these disparate results is not clear, but may reflect different effects of TSA versus

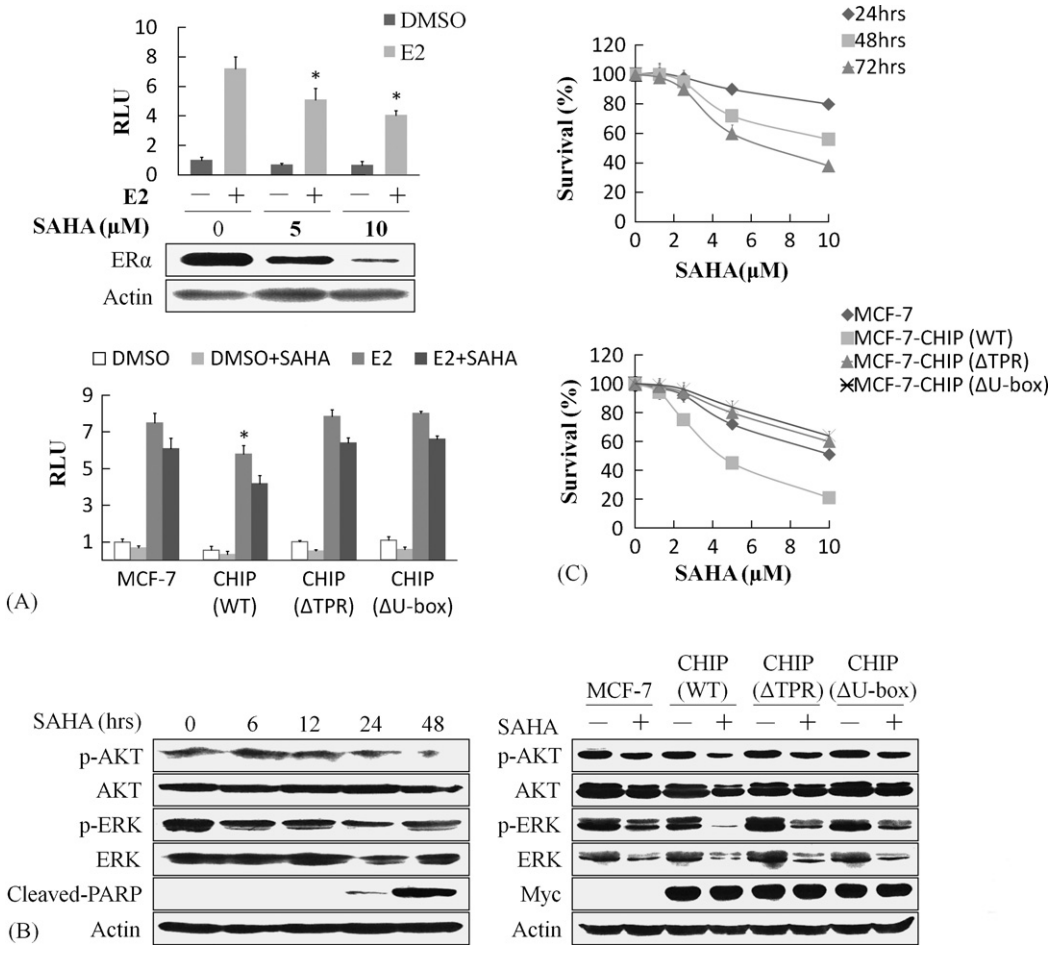

Fig. 4 – SAHA down-regulates ERα transcriptional activity and inactivates survival signaling in MCF-7 cells. (A) After treatment with SAHA (5–10 μM) for 6 h, ERE-Luc plasmid (250 ng) was transiently transfected into MCF-7 cells and the stable transfected cells (MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box)) for 24 h; cells were treated with 10 nM E2 or DMSO as indicated and assayed 24 h later for relative luciferase activity. $P < 0.01$ (student's *t*-test, vs. first group) is indicated by a *. ERα protein was detected by Western blot with anti-ERα. (B) Left figure: MCF-7 cells were treated with SAHA (5 μM) at indicated times. Right figure: MCF-7 cells and the stable transfected cells (MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box)) were treated with SAHA (5 μM) for 48 h. Cell lysate was analyzed by Western blot with antibodies to p-AKT, AKT, p-ERK, ERK, PARP, Myc or actin. (C) Upper figure: MCF-7 cells were treated with SAHA at 1.25–10 μM at indicated times. Lower figure: MCF-7 cells and the stable transfected cells (MCF-7-CHIP (WT), MCF-7-CHIP (ΔTPR) and MCF-7-CHIP (ΔU-box)) were treated with SAHA at 1.25–10 μM for 48 h. Cytotoxicity was evaluated by MTT assays. All experiments were performed in triplicate.

SAHA or attribute to the different doses and times of MG-132 used in the studies.

Recently it has been showed that HDAC inhibitors such as LAQ824, FK228 and SAHA exert their anti-cancer effects by inactivating of Hsp90 molecular chaperone function, impair the chaperone association of Hsp90 with its client proteins, HER2, AR and induce the degradation via the ubiquitin-proteasome pathway. In our studies, the results indicated that SAHA-induced ERα degradation in MCF-7 cells is also associated with inactivation of Hsp90 molecular chaperone function. First, treatment with SAHA induces Hsp90 acetylation, and Hsp90 acetylation is linked to the inactivation of its chaperone function. The disassociation of ERα from the Hsp90 chaperone complex is accompanied with SAHA-induced acetylation of Hsp90. Secondly, besides ERα, other Hsp90 client proteins, such as Raf-1, CDK4 and survivin are also reduced by SAHA. Therefore, these observations suggested that SAHA induces ERα degradation via the ubiquitin-proteasome pathway by inhibiting Hsp90 chaperone function in breast cancer MCF-7 cells. Previous reports have shown that treatment with SAHA induces p21 and cell cycle growth arrest, associated with differentiation and apoptosis of breast cancer cells [13]. Our data indicated that antitumor effects of SAHA are also associated with inhibition of Hsp90 function, which leads to Hsp90 client proteins' degradation.

It is known that molecular chaperones recognize non-native proteins and aid in their correct folding. When it is unsuccessful, the misfolded proteins will be directed to degradation. The mutually exclusive pathways of folding and degradation constitute the cell's protein quality control

system [33]. As recently studies showed, another co-chaperone, CHIP, regulates chaperone function in part by regulating ubiquitin-proteasome pathway and determining whether proteins enter the productive folding pathway or the degradation pathway. CHIP acts as an E3 ubiquitin ligase and promotes ubiquitination of Hsp90 client proteins by a COOH-terminal U-box domain, while binding molecular chaperone Hsp90 or Hsp70 through TPR domain [34]. Recently it has been reported that ERα serves as a substrate for Hsp90/Hsp70-associated CHIP. The association of CHIP with ERα through a chaperone intermediately results in ubiquitination and degradation of ERα. And the presence of Hsp90 inhibitor GA potently stimulates this process. Our data demonstrated that E3 ubiquitin ligase CHIP also mediates SAHA-induced ERα ubiquitination and proteasomal degradation. CHIP overexpression in the presence of SAHA leads to an additive loss of ERα and decreases its transcriptional activity via enhanced ERα ubiquitination. The accelerate degradation of ERα further mediates inactivation of survival signaling pathways and enhances SAHA-induced cytotoxicity. The function of CHIP on mediating ERα degradation is dependent on both the TPR domain and the U-box E3 ligase activity, as transfection with either CHIP (ΔTPR) or CHIP (ΔU-box) has no effect on SAHA-induced ERα degradation or cytotoxicity. Taken together, we concluded that E3 ubiquitin ligase CHIP is necessary for ERα degradation, following Hsp90 inhibition by SAHA. These findings are consistent with previous studies, which showed that overexpression of CHIP by transient transfection enhances ERα degradation [10].

Although tamoxifen has been the standard endocrine treatment for breast cancer for many years, its extended use may be associated with the development of tamoxifen resistance [3]. The potential mechanisms of endocrine resistance are not fully clarified, but evidence suggested that complex interactions between ERα and growth factor signaling pathways are involved. The introduction of new endocrine agents that can induce ERα depletion and block growth signaling pathways, may improve the therapeutic options for women with endocrine-resistant breast cancer. Here, we demonstrated that SAHA, a HDAC inhibitor, induces rapid depletion of ERα protein, resulting in repression of the ERα-dependent transcriptional function. Consistent with our results, Reid and his colleagues reported that another HDAC inhibitor, TSA, has the similar effect on influencing estrogen-dependent transcription [35]. However, their data showed that TSA-induced ERα clearance is due to reduction of the steady-state level of ERα mRNA in MCF-7 cells and consequently suppress the transactivation activity of ERα. In contrast, our results showed at 6 h of SAHA treatment, the protein level of ERα decreased markedly accompanied with inhibition of ERα-dependent transcriptional activation, but with slight down-regulation of ERα mRNA level, suggesting that SAHA-induced ERα transcriptional inactivation is mainly caused by ERα degradation, but not reduction of ERα mRNA. Our results implied that inhibition of ERα-dependent transcriptional activation by SAHA could provide a potentially important strategy to develop new therapeutic agents against breast cancer. Activation of EGFR and HER2 receptors results in stimulation of several signaling pathways including mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways. It has been reported that ERα can be phosphorylated and activated by these protein kinases; increased bidirectional crosstalk between ERα and EGFR signaling pathways is thought to contribute to the development of tamoxifen resistance [6]. In our study, we demonstrated that SAHA could reduce MAPK and PI3K/AKT signaling activity and inhibit the growth of MCF-7 cells. Our data indicated that the use of agents such as SAHA to degrade ERα protein level and abrogate survival signaling pathways may lead to improvements in the prevention of endocrine resistance. Our findings also indicated that CHIP can mediate down-regulation of ERα transcriptional activity and inhibition of survival signaling in MCF-7 cells caused by SAHA, which implied that CHIP may play an important role in HDAC inhibitors-induced antitumor effects.

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