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Hypoxia가 Hsp90의 기능과 변화에 미치는 역할

The Role of Hypoxia in the Function and Modification of Hsp90

2015년 2월

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ABSTRACT

The Role of Hypoxia in the Function and Modification of Hsp90

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Hypoxia is an essential factor of cancer progression. Several proteins, including clients of heat shock protein 90 (Hsp90), are activated/deactivated under hypoxic conditions, leading to changes in tumor microenvironment, metabolism, and response to anticancer therapies. Hsp90 is a chaperone protein that stabilizes a number of proteins required for tumor growth, angiogenesis, and metastasis by assisting proper folding of the proteins. Hsp90 chaperone function is fine-tuned by posttranslational modifications, including acetylation, phosphorylation, S-nitrosylation, oxidation and ubiquitination. HDAC inhibition has shown to increase Hsp90 acetylation while simultaneously destabilizing Hsp90 interaction with several client proteins, including ErbB2, Src, and Hif1α. Deacetylases and acetylases are balanced to regulate this modification. However, little is known about HDACs that modify Hsp90 under hypoxia and acetylases which directly transfer acetylation on Hsp90. Here, I demonstrate that hypoxia increases Hsp90 acetylation and its function by modulating HDAC1, HDAC3, and ARD1 functions,
resulting in the increased interaction between MEK and Hsp90 and activation of MEK and its downstream proteins. Ectopic overexpression of HDAC1 and HDAC3 decreases the interaction of Hsp90 with MEK under hypoxia. Through pulldown, immunoprecipitation, and \textit{in vitro} acetylation assays, I identify that ARD1 mediates interaction with and acetylation of the middle domain of Hsp90. Silencing ARD1 by transfection with siRNA attenuates the interaction between MEK and Hsp90 under hypoxic conditions. ARD1-mediated Hsp90 acetylation and subsequent increase in MEK/FAK signaling contribute to NSCLC migration under hypoxia. Inhibition of ARD1 or overexpression of HDAC1 and HDAC3 significantly reduce the NSCLC cell migration potential. These results suggest that the balanced actions of HDAC1, HDAC3, and ARD1 regulate Hsp90 acetylation and NSCLC migration, supporting the use of epigenetic modulators for regulating NSCLC migration under hypoxia.

Keywords: Hypoxia, Hsp90, Acetylation, HDACs, ARD1, Migration
Student Number: 2013-20207
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INTRODUCTION

Oxygen limitation is one of the common characteristics of solid tumors. Blood vessels in tumor tissues cannot support the demand of oxygen as the tumors grow rapidly. Necrosis in tumor tissues occurs when the distance between the cells and vessels is more than 200 nm (Bussink et al., 2003). Both normal and cancer cells are damaged under hypoxic conditions; however, the cancer cells overcome this challenging environment with angiogenesis, invasion, and metastasis (Charron et al., 2009; Kato et al., 2004; Kim et al., 2001; Wu et al., 2011 ). Many proteins are involved in helping the cells survive under hypoxia such as Heat-shock protein 90 (Hsp90), Histone deacetylase (HDAC) families, Focal Adhesion Kinase (FAK), and Hypoxia Inducible Factor 1 α (HIF1α), etc.

Hsp90 is a chaperone protein that has an important role for proper folding of a large number of client proteins. Hsp90 is known to be overexpressed in cancer cells and is controlled by many cellular stresses such as nutrient and oxygen deprivation, which results in making new vessels to avoid cell death (Moser et al., 2009; Neckers et al., 2012). Therefore, it has magnified as a therapeutic target for cancer therapies. In addition to the upregulation of Hsp90 expression, posttranslational modifications such as acetylation, phosphorylation, S-nitrosylation, oxidation, and ubiquitination have also been identified to change the chaperone functions (Trepel et al., 2010; Figure 1). Phosphorylation on threonine or tyrosine leads to dissociation of interaction between Hsp90 and client proteins (Xu et al., 2012). In many cases, acetylation on lysine residues negatively controls chaperone functions. Mutational study shows that acetylation attenuates the interaction of Hsp90 with cochaperones, which destabilizes several client proteins such as kinase proteins and transcription factors (Scroggins et al., 2007; Yang et al.,
2008). However, other findings indicate that hyperacetylated Hsp90 helps glucocorticoid receptors have a proper function as a transcription activator (Kovacs et al., 2005). These findings imply that more than one acetylation site mediate the chaperone function of Hsp90, and the consequences of these modifications have to be evaluated.

HDACs also play a critical role under hypoxia to help cells evade necrosis. HDAC family is classified into 4 groups: I -IV. Among the identified 18 HDACs, HDAC1, 2, 3, and 7, class I and II proteins, have been examined to be regulated by hypoxia (Charron et al., 2009; Kato et al., 2004; Kim et al., 2001; Wu et al., 2011). The regulated HDACs under hypoxic conditions control the cellular behaviors such as angiogenesis and Epithelial-Mesenchymal Transition (EMT).

HDACs have identified to acetylate both histone and non-histone proteins such as Hsp90 and tubulin. Of the HDACs, HDAC1, 3, and 6 have been investigated as key regulators of Hsp90. Blockage of HDAC1 and 3 with HDAC inhibitors (HDACi) including TSA and MS-275 increases acetylation of Hsp90 (Bali et al., 2005; Nishioka et al., 2008; Kovacs et al., 2005). Moreover, HDAC6 modifies acetylation at K294, which regulates its chaperone activity (Scroggins et al., 2007). However, as reviewed in Scroggins paper, HDACs are not the only modulators of Hsp90 acetylation (2007). Acetyltransferases that function in the opposite way of the deacetylases may modify Hsp90 acetylation. Studies with \textit{Saccharomyces cerevisiae} have showed that N-acetyltransferase 10 (Naa10p or ARD1) is essential enzyme for its life cycle (Lim et al., 2006). Mouse and human homologous of yeast ARD1 have also identified to regulate several cellular functions such as proliferation and differentiation (Fisher et al., 2004). Of mammalian acetyltransferases, ARD1 is known as a novel type of enzyme which
can acetylate not only at serine, threonine, glycine, and alanine residues at amino
terminus of proteins but also at lysine residues within proteins (Arnesen et al.,
2005; Polevoda et al., 2002), which means that ARD1 performs both
cotranslational and posttranslational acetylation (Gautschi et al., 2003). In addition,
previous researches have shown ARD1 function in hypoxic response by acetylating
HIF1α. Lys 532 of HIF1α is acetylated by ARD1, which leads to the degradation
of HIF1α (Jeong et al., 2002). However, other studies report that ARD1 has little
relationship with hypoxia and HIF1α (Bilton et al., 2005). These opposite findings
show the need of additional study about ARD1 and hypoxia.
In the present study, I investigated that hypoxia increased Hsp90 acetylation and
its chaperone activity by regulating HDAC1, HDAC3, and ARD1. These three
modulators mediated the binding of MEK to Hsp90, which results in
phosphorylation of MEK and its downstream protein, FAK. The activation of this
MEK/FAK pathway leads to increase of migration in NSCLC cells under hypoxic
conditions.
Figure 1. Cochaperones and posttranslation modifications regulate Hsp90 chaperone function
MATERIALS AND METHODS

Cell and Cell cultures, reagents.

H1299, 226Br, H460 (human Non-small-cell lung cancer (NSCLC), and UMSCC 38 (human head and neck squamous cell carcinoma) cell lines were cultured in RPMI 1640 (WelGene, Daegu, Korea) with 10% FBS and 1% antibiotics. Antibodies against MEK, pMEK (S217/221), Erk, pErk (T202/Y204), AKT, pAKT (S473), pAKT (T308), Tubulin, pRaf (S338), and HDAC3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-ARD1, Actin, Flag, Raf, ACK (anti-pan-acetyl lysine), HDAC1, HA, GFP, His, and Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Hsp90, Hsp70, and HOP from Stressgen (Victoria, BC, Canada), anti-FAK from BC Pharmingen (San Diego, CA, USA), and anti-pFAK from Biosource (Camarillo, CA, USA).

Western blot and Coimmunoprecipitation analysis

Whole cell lysates were prepared with ice-cold lysis buffer [20 mM HEPES (pH7.4), 120 mM NaCl, 0.5 mM EDTA, 5% Glycerol, 1 mM DTT, 0.5% NP-40, protease inhibitor]. For coimmunoprecipitation, 250–800 µg of total cell lysates were incubated with antibody (250–800 ng) at 4°C. Protein G-agarose beads were added and the mixtures were then washed two times with the same buffer and two times with PBS. For western blot analysis, equivalent amounts of protein (8–15 µg) were separated on 6 to 10% SDS-PAGE gel. The blot was incubated with primary antibodies in TBST containing 3% bovine serum albumin. The membrane was then washed three times with TBST and incubated with the
appropriated secondary antibodies. The signal was visualized using ECL solution (Thermoscientific INC, Bremen, Germany).

**Adenoviral studies**

Adenoviral vectors expressing hemagglutinin (HA)-tagged Hsp90 is prepared as previously described (Oh et al., 2007). H1299, H460, 226Br, and UMSCC38 cells were infected with EV-ad or adenovirus expressing a full-length human Hsp90 for 1 day and were subsequently incubated normoxic (20% O₂) or hypoxic (1% O₂) conditions for 6 to 12 hrs.

**HDAC colorimetric activity assay**

HDAC Activity Colorimetric Assay kit (Biovision, CA, USA) was used according to the manufacturer's protocol. Briefly, 95 μg of cell lysates was diluted in a final volume of 85 μL of ddH2O and placed in a 96-well plate. 10 μL of the 10X assay buffer was transferred to each well followed by 5 μL of the HDAC colorimetric substrate. The plate was incubated at 37 °C for 1 hr. 10 μL of Lysine Developer was then added, and the mixture was incubated at 37 °C for 30 min. The prepared sample was read in an ELISA plate reader at 405 nm.

**Purification of his-tagged proteins**

His-tagged Hsp90 domains were expressed in *Escherichia coli* strain BL21 and cultured in Luria-Bertani (LB) medium at 37 °C. Cells were lysed with a lysis buffer including 50 mM Tris (pH8.0), 150 mM NaCl, 1% TritonX-100, 1% glycerol, and protease inhibitors by sonication. After centrifugation, the lysate was
mixed with Ni-NTA agarose beads (Qiagen, Valencia, CA, USA). The beads were then washed five times with PBST and three times with PBS.

**Pulldown assay**

500 µg of H1299 cellular protein was added to each peptide of His-Hsp90 domains with Ni-NTA agarose beads. The mixture was rotated at 4 °C for 1 hr and washed three times with PBST and two times with PBS. 5X sample buffer were added and the samples were separated on SDS-PAGE gels.

**Small interfering RNA transfection**

H1299 and 226Br cells were transfected with siRNA against ARD1 (Integrated DNA Technologies, Coralville, IA, USA) using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. At 48hr after siRNA transfection, cells were incubated under normoxic or hypoxic conditions for 6hrs.

**Immunocytochemistry and Microscopy**

H1299 cells were seeded on cover slips. After being incubated overnight, the cells were cotransfected with GFPARD1 and Myc-Hsp90. Cold methanol was used to fix the cells for 30 min and then washed three times with PBS. To reduce nonspecific binding, the fixed cells were incubated with 3% bovine serum albumin. For antibody incubation, anti-Myc antibody (1:100 in 3% BSA) and Alexa Fluor 594 (1:1000, Invitrogen) were applied as primary and secondary antibodies, respectively. Fluorescent signals, green and red, were detected using a
confocal microscope.

**Acetylation assay**

ARD1 protein was immunoprecipitated from 500 µg of cell lysates using anti-ARD1 antibody and protein G-agarose. Immunoprecipitates were washed twice with PBST and once with acetyl-transferase assay buffer [50 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 10 mM MgCl₂, 0.01 mM Acetyl CoA]. His-Hsp90 peptides were used at 2 µg/reaction. Acetylation reactions were incubated for 45 min at 30 °C. Acetylated His-Hsp90 peptides were identified by immunoblotting with ACK antibody.

**Wound healing assay**

H1299 cells were transfected with FlagHDAC1, FlagHDAC3 or siRNA against ARD1 using Fugene 6 or lipofectamine 2000. At 24 hr after transfection, cells were seeded again at 5X10⁵ cells per well of 12-well plates. 1 day after seeding, the monolayer surface was scratched with a tip. The cells were incubated under normoxia or hypoxia for 12 hrs.
RESULTS

Hypoxia modifies Hsp90 acetylation status and its function

I first determined whether hypoxia regulates acetylation of Hsp90 in cancer cells by using multiple NSCLC and HNSCC cell lines after infection with Hsp90-expressing adenovirus (Ad-HA-Hsp90). Under hypoxic conditions, both endogenous and virus-induced Hsp90 were acetylated in indicated cancer cells (Figure 2A and 2B). Because acetylation of endogenous Hsp90 increased in all indicated NSCLC cell lines within 6 hr of hypoxia, the following experiments were mostly performed under hypoxic conditions for 6 hrs.

To study the impact of hypoxia on Hsp90 function, I immunoprecipitated cochaperone-Hsp90 complex with anti-Hsp70 and anti-HOP antibodies. Exposure to hypoxia resulted in enhanced binding of both Hsp70 and HOP with Hsp90 (Figure 3A and 3B). Immunoprecipitation assays with client proteins were also conducted. The interaction of Hsp90 with client proteins such as AKT, MEK, and Raf significantly increased under hypoxic conditions, but the total expression level of these client proteins were not changed (Figure 4A and 4B). Western blot analysis was performed to investigate whether the client proteins were activated as Hsp90 strongly interacted with the clients under hypoxic conditions. It was previously shown that MEK and AKT are activated under hypoxia (Beitner et al., 2001; Whelan et al., 2010; Yokoi et al., 2004). However, MEK was only confirmed to increase its phosphorylation status when AKT and Raf showed almost no change under hypoxia (Figure 4A and 4B). These findings implied that hyperacetylated Hsp90 under hypoxic conditions regulated activation of a specific client protein, MEK, by modulating its chaperone function.
Figure 2. Hsp90 is acetylated under hypoxic conditions.

A. Lung cancer cells, H1299, 226Br, and H460, were immunoprecipitated with anti-pan-acetyl antibody (ACK). Acetylation of endogenous Hsp90 was detected under hypoxia.

B. Cells were treated with adenoviral vectors expressing HA-tagged Hsp90. After 24 hr, cells were incubated under normoxic and hypoxic conditions for 12 hrs. Exogenous Hsp90 acetylation was detected.
Figure 3. The interaction between Hsp90 and cochaperones increases under hypoxia.

A. H1299 cells were incubated under hypoxia and lysed. Cells were immunoprecipitated with anti-Hsp70, and associated Hsp90 was detected by immunoblotting.

B. H1299 cells were immunoprecipitated with anti-HOP, and associated Hsp90 was detected by immunoblotting.
Figure 4. The interaction between Hsp90 and client proteins increases under hypoxia.

A. H1299 cells were exposed to hypoxia and lysed. Cells were immunoprecipitated with anti-AKT, and associated Hsp90 was detected by immunoblotting. AKT phosphorylation was also detected.

B. H1299 cells were immunoprecipitated with anti-MEK and Raf, and associated Hsp90 was detected by immunoblotting. MEK phosphorylation was also detected.
Acetylation and function of Hsp90 are regulated by HDAC1 and HDAC3

HDAC activity in hepatocytes was shown to decrease under hypoxic conditions in a time dependent manner (Evankovich et al., 2010). Consistent with this, my findings showed that HDAC activity in H1299 cells decreased about 20% at 12 hr of hypoxia (Figure 5A). But the expression levels of HDAC1 and HDAC3 were not changed under hypoxia (Figure 5B). Of the HDAC families, Hsp90 acetylation is known to be regulated by HDAC1, 3, and 6 (Ha et al., 2012; Kovacs et al., 2005; Nishioka et al., 2008; Scroggins et al., 2007). Ectopic overexpression of HDAC1 and HDAC3 reduced Hsp90 acetylation under hypoxic conditions (Figure 6A). As HDAC6 was previously identified to mediate acetylation of Hsp90 under normoxic conditions, immunoprecipitation result with overexpression of HDAC6 showed decreased Hsp90 acetylation under normoxia (Figure 6B). However, Hsp90 was still hyperacetylated under hypoxia even with overexpression of HDAC6 (Figure 6B).

I next investigated whether HDAC1 and HDAC3 could regulate the Hsp90 chaperone function. Overexpression of HDAC1 and HDAC3 weakened the association of both Hsp70 and MEK with Hsp90 under hypoxia (Figure 7A and 7B) However, the bindings of both AKT and Raf with Hsp90 were not affected by overexpression of HDAC1 (Figure 7C) To determine if the increased interaction between Hsp90 and MEK mediated activation of MEK and its downstream protein, western blot analysis was performed. According to the previous studies, FAK signaling pathway was shown to be regulated by MEK (Acton et al., 2013), and FAK was phosphorylated under hypoxic conditions (Lee et al., 2010). Consistent with this, FAK was activated under hypoxia, and
PD98059, MEK inhibitor, decreased FAK phosphorylation under hypoxic conditions (Figure 8A). These activated MEK and FAK were dephosphorylated by ectopic overexpression of HDAC1 and HDAC3 (Figure 8B). These results indicated that Hsp90 acetylation and its chaperone functions were mediated by HDAC1 and HDAC3.
Figure 5. HDAC activity decreases under hypoxic conditions.

A. H1299 cells were incubated under hypoxia for indicated hours. The cells were lysed and whole cell lysates were analyzed for HDAC activity.

B. H1299 cells were exposed to hypoxia for 6, 9, and 12 hrs. Immunoblotting was performed to detect HDAC1 and HDAC3.
Figure 6. HDAC1 and HDAC3 control acetylation of Hsp90 under hypoxia.

A. H1299 cells were transfected with indicated FlagHDAC1 and FlagHDAC3 constructs. Cells were exposed to hypoxia for 6 hrs. ACK antibody was used for immunoprecipitation, and endogenous Hsp90 was detected by immunoblotting.

B. H1299 cells were transfected with FlagHDAC6 plasmids. Cells were treated as indicated in Figure 6A.
Figure 7. HDAC1 and HDAC3 mediate interaction between Hsp90 and MEK.

A. H1299 cells were transfected with FlagHDAC1 and FlagHDAC3, and exposed to hypoxia for 6 hrs. Cellular proteins were immunoprecipitated with anti-Hsp70 antibody, and interacted Hsp90 was detected.

B. H1299 cells were transfected and treated as indicated above. MEK was
immunoprecipitated, and Hsp90 association was detected.

C. H1299 cells were transfected with indicated FlagHDAC1, and incubated under hypoxia for 6 hrs. Endogenous AKT and Raf were immunoprecipitated, and Hsp90 binding was detected by immunoblotting.
Figure 8. Phosphorylation of MEK and FAK is controlled by HDAC1 and HDAC3 under hypoxia.

A. H1299 cells were treated with DMSO or 50 µM of PD98059. After 3 hr of treatment, cells were exposed to hypoxia for 6 hrs. Indicated proteins were detected by immunoblotting.

B. H1299 cells were transfected with FlagHDAC1 or FlagHDAC3, and incubated under hypoxia for 6 hrs. The cell lysates were immunoblotted.
ARD1 regulates acetylation of Hsp90 and its function.

In addition to deacetylases, acetyltransferases which might regulate Hsp90 acetylation under hypoxia were investigated. Of several kinds of mammalian acetylases, ARD1 was shown to be regulated in response to hypoxia (Kalvik et al., 2012). With ectopic overexpression of ARD1, acetylation of Hsp90 dramatically increased under hypoxic conditions (Figure 9A). To ensure these findings, siRNA against ARD1 was introduced to H1299 and 226Br cells. Reduced Hsp90 acetylation with siARD1 was detected under hypoxia (Figure 9B).

Next, I studied whether ARD1 could regulate the function of Hsp90. As shown in Figure 10A, increased interactions of Hsp90 with cochaperone proteins under hypoxic conditions were abolished when ARD1 was silenced. The binding of Hsp90 to MEK with siRNA against ARD1 also decreased under hypoxia (Figure 10B). Moreover, the phosphorylated MEK and FAK under hypoxia were deactivated by knockdown of ARD1 (Figure 10B). These findings implied that ARD1 regulated the binding of Hsp90 to cochaperones and clients, and mediated MEK/FAK signalling pathway.
Figure 9. Acetylation of Hsp90 is regulated by ARD1.

A. H1299 cells were transfected with indicated GFPARD1, and incubated under hypoxic conditions for 12 hrs. The cell lysates were immunoprecipitated with ACK antibody, and Hsp90 was detected by immunoblotting.

B. 226Br and H1299 cells were treated with si-con or si-ARD1, and exposed to hypoxia for 6 hrs. ACK antibody was used for immunoprecipitation, and Hsp90 was detected.
Figure 10. ARD1 enhances Hsp90 chaperone activity.

A. H1299 cells were treated with si-con and si-ARD1 and incubated under hypoxic conditions for 6 hrs. Immunoprecipitation was performed with anti-Hsp70 and anti-HOP antibodies, and associated Hsp90 was detected.

B. Cells were treated as indicated above. MEK was immunoprecipitated and associated Hsp90 was detected. FAK and MEK phosphorylation were also detected.
ARD1 interacts with and directly acetylates Hsp90

I further researched that Hsp90 could interact with ARD1. Under hypoxic conditions, the interaction between endogenous Hsp90 and ARD1 dramatically increased (Figure 11A). This enhanced binding was confirmed between exogenous ARD1 and endogenous Hsp90 proteins (Figure 11B). The immunoprecipitation assay and confocal image also demonstrated the interaction between Hsp90 and ARD1 proteins in exogenous level in H1299 cell lines (Figure 11C and 11D). The increased co-localization of these two proteins was detected under hypoxia (Figure 11D). These results implied that Hsp90 and ARD1 appear to interact under hypoxic conditions.

To confirm the interaction between Hsp90 and ARD1 by an in vitro pulldown assay, I prepared three domains of Hsp90 recombinant peptides: N-terminal, middle, and C-terminal domains. Figure 12A showed that middle domain of Hsp90 revealed significantly higher affinity to ARD1 than N-terminal and C-terminal domains. Immunoprecipitation assay was conducted to confirm this interaction. Plasmids expressing full length and each domain of Hsp90 were introduced to NSCLC cell lines. Endogenous ARD1 showed stronger association with middle domain of Hsp90 than other domains (Figure 12B). These data indicated that the middle domain of Hsp90 was critical for its binding with ARD1.

To test if ARD1 acetylates Hsp90 directly, in vitro acetylation assay was performed using recombinant peptides. His-Hsp90 middle domain was shown to be acetylated under hypoxia (Figure 12C). This implied that ARD1 acetylated at least one lysine residue located within middle domain of Hsp90.
Figure 11. ARD1 interacts with Hsp90.

A. H1299 cells were exposed to hypoxia for 12 and 24 hrs. Cells were immunoprecipitated with anti-ARD1 antibody. Associated endogenous Hsp90 was detected.
B. H1299 cells were transfected with GFPARD1 constructs. GFPARD1 was immunoprecipitated, and endogenous Hsp90 was detected.

C. H1299, H460, UMSCC38, and 226Br cells were treated with adenoviral vectors expressing HA-tagged Hsp90 and transfected with GFPARD1 constructs. The cells were incubated under hypoxia for 12 hrs. GFPARD1 was immunoprecipitated, and associated exogenous HA-Hsp90 was detected.

D. H1299 cells were cotransfected with GFPARD1 and Myc-Hsp90 constructs and stained with anti-Myc antibodies and DAPI.
Figure 12. ARD1 associates with and directly acetylates middle domain of Hsp90.

A. Recombinant his-tagged Hsp90 domains bound with Ni-NTA-agarose beads were mixed with H1299 cellular proteins that were exposed to 12hr of hypoxia. Associated ARD1 was detected by immunoblotting.

B. H1299 and 226Br cells were co-transfected with GFPARD1 and each myc-tagged Hsp90 domain. Myc-hsp90 was immunoprecipitated and
GFPARD1 was detected. And GFPARD1 was immunoprecipitated and immunoblotting was used to detect myc-Hsp90.

C. Endogenous ARD1 was immunoprecipitated with anti-ARD1 antibody, and incubated with His-Hsp90 middle domain and acetyl-CoA at 30 °C. The lysine acetylation of His-Hsp90 was detected using ACK antibody.
HDAC1, HDAC3, and ARD1 control cell migration under hypoxia.

Under hypoxia, cell migration was shown to increase by MEK and FAK signaling pathway (Lee et al., 2010; Lester et al., 2005). Given that HDAC1, HDAC3, and ARD1 regulated activation of MEK and FAK, I suggested that overexpression of HDAC1 and HDAC3 and knockdown of ARD1 would inhibit migration of NSCLC cells. To verify this, wound healing assay was performed. As shown in Figure 13, overexpressed HDAC1 and HDAC3 weakened the cell migration under hypoxic conditions. This pattern of migration was also shown when siRNA against ARD1 was treated (Figure 13). Taken together, modulators of Hsp90 acetylation mediated NSCLC migration by regulating MEK/FAK pathway under hypoxia.
Figure 13. HDAC1, HDAC3, and ARD1 regulate cell migration under hypoxia.

H1299 cells were transfected with FlagHDAC1 and FlagHDAC3 constructs, and were treated with siARD1. Wound healing assay was conducted. Wound was performed after confluence, and cells were exposed to Hypoxia.
Hypoxia regulates several kinds of proteins that lead to modifications of cellular behaviors. Most of these proteins are clients of Hsp90 such as AKT, MEK and Raf, and the stability and activation of these clients are regulated by posttranslational modifications of Hsp90 (Grbovic et al., 2006; Scaltriti et al., 2012). Recently, many studies were performed on Hsp90 acetylation. Several findings concluded that acetylation at K294 modulated by HDAC6 has been shown to abolish Hsp90 chaperone activity and reduce the viability of yeasts (Scroggins et al., 2007). In addition to HDAC6, other HDAC families also have been identified to modify Hsp90 acetylation. According to the research conducted by Yang et al., there are seven possible lysine residues of Hsp90 that can be acetylated, which are regulated by HDAC inhibitors (2008). HDAC activities and expressions have been shown to regulate under hypoxic conditions (Charron et al., 2009; Evankovich et al., 2010; Wu et al., 2011). These previous data indicate a potential relationship among hypoxia, Hsp90, and HDACs.

In this paper, I have demonstrated that hypoxia increases Hsp90 acetylation and its chaperone activity. Both cochaperones and client proteins show stronger interaction with Hsp90 under hypoxic conditions than in normoxic conditions. According to previous report, hypoxia does not alter expression of AKT and MEK but increases phosphorylation status (Zeng et al., 2010). I confirm that MEK is only activated under hypoxia in a time dependent manner. This suggests that the increased Hsp90 function mediates to activate MEK under hypoxic conditions. Previous study shows that MS-275, HDAC1 inhibitor, regulates Hsp90 acetylation and its function in AML cells (Nishioka et al., 2008). Recently, HDAC3 is shown to acetylate and interact with Hsp90 (Ha et al., 2012). My findings in this paper
identify that these HDAC1 and HDAC3 are working as modulators of Hsp90 acetylation under hypoxia as well as normoxia. However, the most well-known deacetylase of Hsp90, HDAC6, does not alter Hsp90 acetylation under hypoxic conditions (Figure 6B). This suggests that HDAC1 and HDAC3 are the mediators of Hsp90 acetylation under hypoxia. These two HDACs mediate the interaction between Hsp90 and MEK as well. Yet, the bindings with other client proteins are not changed under hypoxia when HDAC1 and HDAC3 are overexpressed (Figure 7C). Even though hypoxia increases the interaction of both AKT and Raf with Hsp90, it does not affect stability and activity of those clients. Taken together, the results indicate that increased association of Hsp90 with AKT and Raf may affect different signaling pathways and be regulated by other HDACs under hypoxia.

ARD1 is an enzyme that transfers acetyl group from acetyl-CoA to multiple residues in proteins through cotranslational and posttranslational modification (Arnesen et al., 2005; Polevoda et al., 2002). ARD1 is also known to be regulated by cellular stress such as oxidative stress (ROS) and hypoxia by regulating methionine sulfoxide reductase A (MSRA) and HIF1α, respectively (Kalvik et al., 2012; Shin et al., 2014). Here, the relationship between ARD1 and hypoxia was confirmed by immunoprecipitation assay. ARD1 regulates acetylation of and interacts with Hsp90 under hypoxic conditions, but the expression of ARD1 is not changed. I then focused on the enhanced interaction between Hsp90 and ARD1 under hypoxia to research which domain of Hsp90 is responsible for this binding. Middle domain of Hsp90 has shown to be vital for the interaction with client proteins. Moreover, Scroggins et al. have identified that HDAC6 binds to the middle domain of Hsp90 and acetylates K294 in this domain (2007). Consistent with this finding, pulldown, immunoprecipitation, and in vitro
acetylation assays showed that ARD1 associates with the Hsp90 middle domain and directly transfers acetyl group to one or more lysine residues within the middle domain. According to Muller et al., C-terminal phosphorylation of Hsp90 and Hsp70 led to the increased interaction between these two proteins and the proper chaperone activity (2012). Highly conserved sequence EEVD-COOH is at the end of Hsp90 and Hsp70, and it binds to cochaperones containing tetratricopeptide repeat (TPR) such as HOP (Muller et al., 2012). I demonstrated that the knockdown of ARD1 disrupts the binding of both Hsp70 and HOP to Hsp90 under hypoxia. In addition, MEK and Hsp90 interaction was attenuated, and phosphorylation of MEK and FAK was reduced when ARD1 level decreases. This suggests that the binding of ARD1 to the Hsp90 middle domain affects protein-protein interactions at the c-terminal domain as well as the middle domain, and this interaction is important for Hsp90 chaperone activity under hypoxia.

MEK and FAK are responsible for increased migration in hypoxic conditions (Lee et al., 2010; Lester et al., 2005). These two groups have researched MEK and FAK involvement in cell migration independently. My findings in this paper suggest that FAK is phosphorylated by MEK activation, which increases migration under hypoxic conditions. Migration of lung cancer cells decreases under hypoxia when HDAC3 is overexpressed. This finding is similar to a recent study of Kim (Kim et al., 2010). Taken together, phosphorylated MEK and FAK resulting from HDAC1, HDAC3, and ARD1 regulate cell migration under hypoxia.

In summary, the findings of this paper demonstrate that Hsp90 acetylation and its function are enhanced under hypoxic conditions. HDAC1, HDAC3, and ARD1 are the regulators of Hsp90 acetylation under hypoxia, which leads to the activation of MEK/FAK pathway. As a result, hypoxia promotes H1299 lung cancer cell
migration by the MEK/FAK-dependent pathway (Figure 14).
Figure 14. Mechanism of acetylated Hsp90-mediated migration in cancer cells under hypoxic conditions.
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국문초록

저산소증은 세포내 여러 단백질들의 활성화 또는 비활성화를 일으켜 암의 진행과정에 밀접하게 관여하는 요소로 알려져 있다. 이 중에 많은 단백질들이 Heat shock protein 90 (Hsp90)의 client로 알려져 있는데 이들은 암의 성장, 혈관 신생, 암의 전이 등 많은 부분에 걸쳐 암의 진행을 주도하고 있다. Hsp90은 client 단백질들의 proper folding을 도와주는 chaperone 단백질으로써 Hsp90의 이러한 활성은 posttranslation modification인 아세틸화, 인산화, 유비퀴틴화등의 반응에 의해서 조절된다고 알려져 있다. 이중에 Hsp90의 아세틸화 반응은 Histone deacetylases (HDACs) 에 의해 조절되며, 이로 인하여 ErbB2, Src, Hif1α와 같은 client들의 안정성이 떨어진다고 밝혀져있다. Deacetylase는 이와 반대의 역할을 담당하는 acetylases와의 균형에 맞춰 단백질의 acetylation을 조절한다고 하는데 HDAC과는 달리 Hsp90을 조절하는 acetylases는 알려진 바가 적다. 이에 본 논문에서는 Hsp90의 아세틸화 반응을 조절한다고 알려진 HDAC들이 저산소 상태에서도 같은 역할을 할 수 있는지, 그리고 acetylases중에는 어떠한 단백질이 Hsp90의 modulator로 작용하는지 알아보고자 하였다. 먼저 폐암세포주와 두경부암세포주에서 Hsp90의 아세틸화 반응이 저산소 상태에서 증가함을 확인할 수 있었다. 아세틸화의 증가와 함께 저산소 상태에서 client인 MEK과 cochaperone인 HOP, Hsp70과 Hsp90의 결합이 증가함을 확인하였다. 이러한 증가는 HDAC1과 HDAC3가 과발현 되었을 때 또는 ARD1을 siRNA를 통해 knockdown시켰을 때 조절이 됐을 알 수 있었다. 저산소 상태에서 증가한 MEK과 Hsp90의 결합을 통해 MEK이 활성화 되었음을 알았고, MEK의 하위신호전달 단백질인 Focal Adhesion Kinase (FAK) 역시 인산화 반응이 증가한 것을 알게 되었다. 이러한 MEK/FAK 신호전달 체계의 활성화로 인해 저산소 상태에서 폐암세포주의 전이가 증가하였다. 이와 같은 연구 결과로서,
저산소 상태에서 HDAC1, HDAC3, 그리고 ARD1의 기능을 통해 Hsp90의 아세틸화와 암세포의 전이가 증가한다는 것을 알 수 있었다.

주요어: 저산소증, Hsp90, 아세틸화 반응, HDACs, ARD1, 암세포 전이
학번: 2013-20207