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Disruption of polyubiquitin gene *Ubc* leads to attenuated resistance against arsenite-induced toxicity in mouse embryonic fibroblasts

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ABSTRACT

The polyubiquitin gene *Ubc* is upregulated under oxidative stress induced by arsenite [As(III)]. However, the detailed mechanism of *Ubc* upregulation and the exact role of ubiquitin (Ub) to protect cells against As(III)-induced toxicity remain unknown. Here, we found that $Ubc^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited reduced viability under As(III) exposure, although the Nrf2–Keap1 pathway was activated as a cytoprotective response. Intriguingly, due to the reduced polyubiquitination and delayed onset of degradation of Nrf2 in $Ubc^{-/-}$ MEFs, the basal expression levels of Nrf2 target genes were elevated. As(III)-induced accumulation of Ub conjugates occurred in an Nrf2-independent manner, probably due to cellular stress conditions, including reduced proteasomal activity. Increased cellular Ub levels were essential to polyubiquitinate misfolded proteins generated under As(III) exposure and to degrade them by the proteasome. However, when cellular Ub levels decreased, these misfolded proteins were not efficiently polyubiquitinated, but rather accumulated as large protein aggregates inside the cells, causing cytotoxicity. Furthermore, increased activity of the autophagic pathway to clear these aggregates was not observed in $Ubc^{-/-}$ MEFs. Therefore, reduced viability of $Ubc^{-/-}$ MEFs under As(III) exposure may not be due to dysregulation of the Nrf2–Keap1 pathway, but mostly to reduced efficacy to polyubiquitinate and degrade misfolded protein aggregates.

1. Introduction

Oxidative stress is induced when cellular levels of reactive oxygen species (ROS) outbalance their antioxidant response capacity, which results in perturbation of the cellular redox state by producing peroxides and free radicals that damage DNA, proteins, and lipids [1–5]. Upon exposure to oxidative stress, the cellular stress response pathway is activated to increase the likelihood of survival [6]. Cellular defense against toxicity caused by oxidative stress is achieved by upregulating the cytoprotective antioxidant genes through the activation of mitogen-activated protein kinases (MAPKs) and the pathway involving nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) [7]. Impairment in Nrf2-mediated cellular defense mechanisms is related to various diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases [8–13].

The Nrf2–Keap1 pathway is the major antioxidant response pathway [1–5,14,15]. Nrf2 is a transcription factor that induces the expression of genes harboring antioxidant response element (ARE) in their promoter region [16–20]. Under normal conditions, Nrf2 is polyubiquitinated by the Keap1-Cul3 E3 ligase complex and is rapidly degraded by the 26S proteasome with a half-life of <20 min, thereby maintaining its protein levels quite low in the cytoplasm [21–24]. However, under oxidative or electrophilic stress conditions, the critical Cys residues of Keap1 are modified; therefore, the E3 ligase complex cannot be formed and Nrf2 no longer serves as a target [25–28]. Alternatively, autophagy deficiency or arsenite [As(III)] exposure induces the accumulation of p62, an autophagy adaptor protein, which directly binds to Keap1 and leads to the sequestration of Keap1 to the autophagosome; therefore, E3 ligase complex cannot be formed [29–31]. As a result, Nrf2 is released and becomes stabilized, enters the nucleus, and induces the transcription of target genes that encode detoxifying enzymes and antioxidant proteins. p62 is also a known target of Nrf2, creating a positive feedback loop for prolonged activation of Nrf2 [32]. Although Nrf2 mostly exerts a protective role against cytotoxicity [33], it can also be harmful as cancer cells are also protected by Nrf2. In fact, the constitutive activation of Nrf2 has been reported in various tumors or chemoresistance of cancer cells [7,11,34–36]. Furthermore, As(III) exposure not only leads to prolonged activation of Nrf2 but also to increased levels of ubiquitin (Ub) conjugates [37]. In addition, the polyubiquitin gene *Ubc* is upregulated upon exposure to As(III) [38]. However, the detailed mechanism of *Ubc* upregulation and the exact role of Ub to protect cells under As(III) exposure remain unknown.

Ub is a well conserved eukaryotic protein composed of 76 amino acids [39–41]. As a post-translational modification of the protein

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substrate, the Ub conjugation reaction occurs through the action of E1 activating enzymes, E2 conjugation enzymes, and E3 ligases. Ubiquitination of substrates can be divided into two major classes. Monoubiquitination plays a role in endocytosis and histone modification [42], whereas polyubiquitination plays a role in proteasomal degradation, regulation of the cell cycle, DNA repair, and kinase activation depending on Ub chain linkage [43]. Among these, the most important and well-known role of Ub is the degradation of proteins by targeting them to the 26S proteasome. Due to the diverse roles of Ub in cellular signaling and function, maintaining cellular Ub levels is important for cell survival. In particular, disruption of cellular Ub homeostasis has been thought to influence resistance against oxidative stress, although the detailed mechanism has yet to be identified. Therefore, we investigated the relationship between cellular Ub levels and integrity of the antioxidant response pathway.

First, we found that *Ubc*$^{-/-}$ mouse embryonic fibroblasts (MEFs), in which cellular Ub levels decreased due to the disruption of *Ubc*, exhibited reduced viability under oxidative stress induced by As(III). Based on this observation, we hypothesized that maintenance of cellular Ub levels is important for the antioxidant response, and we tested our hypothesis by investigating whether or not activation of the Nrf2–Keap1 pathway *via tert*-butylhydroquinone (tBHQ) or As(III) exposure is dependent on cellular Ub levels. Next, as it is known that misfolded proteins generated under As(III) exposure are polyubiquitinated to be degraded by the 26S proteasome [44,45], and As(III) is also known to trigger the formation of protein aggregates in yeast [46], we looked into this possibility in MEFs treated with As(III). Misfolded proteins could not be adequately polyubiquitinated in *Ubc*$^{-/-}$ MEFs, potentially resulting in aggregation of these proteins instead of being targeted to the proteasome. In fact, we demonstrated that the number of large protein aggregates increased significantly when cellular Ub levels decreased under As(III) exposure.

Here, we demonstrate that degradation and stabilization of Nrf2 is tightly regulated in MEFs in the absence and presence of oxidative stress, respectively. In contrast, when cellular Ub levels decreased, cellular desensitization against oxidative stress may occur due to the presence of Nrf2 spared from degradation, even under normal or unstressed conditions. However, the overall function of Nrf2 was intact, and impairment in the Nrf2–Keap1 pathway was not observed in *Ubc*$^{-/-}$ MEFs under As(III) exposure. Rather, the Nrf2-independent pathway also plays an important role in increasing cellular Ub levels *via* upregulation of *Ubc* under As(III) exposure, which is required for polyubiquitination and degradation of misfolded proteins. However, when cellular Ub levels decreased, these misfolded proteins accumulated as large protein aggregates with delayed clearance. In sum, the reduced viability of *Ubc*$^{-/-}$ MEFs under oxidative stress induced by As(III) seemed to be mostly due to toxicity caused by the accumulated misfolded protein aggregates.

2. Materials and methods

2.1. Mouse studies

Ubc$^{+/-(eGFP\text{-}puro)}$ (or simply *Ubc*$^{+/-}$), *Ubb*$^{+/-(eGFP\text{-}puro)}$ (or simply *Ubb*$^{+/-}$), *Nrf2*$^{+/-}$, and *Nrf2*$^{-/-}$ mice were maintained in plastic cages with *ad libitum* access to food and water. Breeding pairs of *Nrf2*$^{-/-}$ mice were kindly donated by Dr. Sue Goo Rhee at Ewha Womans University (currently at Yonsei University). All animal procedures were approved by the University of Seoul Institutional Animal Care and Use Committee (UOS IACUC; UOS-091201-1 and UOS-121025-2).

2.2. Isolation and culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were generated from 13.5-day post-coitum mouse embryos, as previously described [47]. Either wild-type or heterozygous (*Ubc*$^{+/-}$ or *Nrf2*$^{+/-}$) MEFs served as a control, as there were no discernable phenotypes when only one *Ubc* or *Nrf2* allele was deleted, as previously described [47–49].

2.3. Cell viability assay

MEFs were treated with the indicated concentration of sodium arsenite ($NaAsO_2$) [As(III)] for 12 h. The cell viability assay was carried out as previously described [47].

2.4. Immunofluorescence analysis and TUNEL assay

For immunofluorescence, MEFs grown on coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature (RT), permeabilized with 0.4% Triton X-100/PBS, and blocked with 3% BSA/PBS for 1 h at RT. Fixed cells were incubated with anti-Ub (FK2; 1:1000, Millipore) and anti-Nrf2 (sc-722; 1:500, Santa Cruz Biotechnology) antibodies at 4 °C overnight, washed with 0.05% Tween 20/PBS, and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000, Invitrogen) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1000, Invitrogen) along with 0.1 μg/ml of DAPI for 1 h at RT. Cells were then mounted using ProLong Gold antifade reagent (Invitrogen) and visualized with a Carl Zeiss AxioImager A2 microscope. The number of Ub-positive puncta and their size were determined by ImageJ particle analysis software (1.47v). Briefly, 8-bit grayscale images were thresholded to highlight all of the particles to be counted. Particles with sizes ranging from 0 to infinity in^2 were counted and divided into four groups based on their size. Extra small puncta (particle area $< 5 \times 10^{-5}$ in^2) were assigned to group 0, small puncta (5×10^{-5} in$^2 \leq$ particle area $< 2.5 \times 10^{-4}$ in^2) were assigned to group 1, medium puncta (2.5×10^{-4} in$^2 \leq$ particle area $< 6.5 \times 10^{-4}$ in^2) were assigned to group 2, and large puncta (particle area $\geq 6.5 \times 10^{-4}$ in^2) were assigned to group 3. To detect $NaAsO_2$-induced apoptosis in MEFs, the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay was performed using an Apoptag® red *in situ* apoptosis detection kit according to the manufacturer's protocol (Millipore).

2.5. Immunoblot analysis and indirect competitive Ub-ELISA

Cell lysates were prepared in RIPA buffer [25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, with 1 mM PMSF, 1 μg/μl aprotinin, and 1 μg/μl leupeptin as protease inhibitors] and incubated on ice for 20–30 min. Total cell lysates were centrifuged at 15,000 ×*g* for 10 min at 4 °C, and the supernatant was then removed to measure protein concentration using the BCA protein assay (Pierce). The total cell lysates (15 μg) were subjected to SDS-PAGE followed by immunoblot detection with anti-GFP (MAB3580; 1:500, Millipore), anti-Ub (MAB1510; 1:500, Millipore), anti-Ub (SPA200; 1:1000, Enzo Life Sciences), anti-Nrf2 (sc-722; 1:1000, Santa Cruz Biotechnology), anti-Keap1 (sc-15246; 1:200, Santa Cruz Biotechnology), anti-Nqo1 (ab34173; 1:3000, Abcam), anti-Hmox1 (#5061; 1:3000, Cell Signaling Technology), anti-Hsp70 (SPA810; 1:1000, Enzo Life Sciences), anti-Hsf1 (SPA901; 1:2000, Enzo Life Sciences), anti-SQSTM1/p62 (#5114; 1:500, Cell Signaling Technology), anti-LC3B (#2775; 1:2000, Cell Signaling Technology), anti-Lamin B (sc-6217; 1:1000, Santa Cruz Biotechnology), or anti-α-tubulin antibody (T6199; 1:5000, Sigma-Aldrich). Based on the type of primary antibodies, we used appropriate secondary antibodies; HRP conjugated goat anti-mouse or anti-rabbit IgG (1:10,000, Enzo Life Sciences) or HRP conjugated donkey anti-goat IgG (1:10,000, Santa Cruz Biotechnology). Immunoreactive complexes were visualized using ECL Plus western blotting substrate (Pierce). The membranes were exposed to X-ray film (Hyperfilm® ECL) and developed. Indirect competitive ELISA was carried out as previously described [47,50,51].