S-glutathionylation of Hsp90 enhances its degradation and correlates with favorable prognosis of breast cancer

Yu-Yin Shih \textsuperscript{a,b}, Hsien-Ya Lin \textsuperscript{b}, Hau-Ming Jan \textsuperscript{b}, Yu-Ju Chen \textsuperscript{c}, Lih-Lih Ong \textsuperscript{b,c,f,g}, Alice Lin-Tsing Yu \textsuperscript{d}, Chun-Hung Lin \textsuperscript{b,c,f,g}

\textsuperscript{a} x-Dimension Center for Medical Research and Translation, China Medical University Hospital, Taichung, 404332, Taiwan
\textsuperscript{b} Institute of Biological Chemistry, Academia Sinica, Taipei, 11529, Taiwan
\textsuperscript{c} Institute of Chemistry, Academia Sinica, Taipei, 11529, Taiwan
\textsuperscript{d} Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, Taoyuan, 33305, Taiwan
\textsuperscript{e} Department of Chemistry and Institute of Biochemical Sciences, National Taiwan University, Taipei, 10617, Taiwan
\textsuperscript{f} Department of Applied Chemistry at National Yang Ming Chiao Tung University, Hsin-Chu, 30009, Taiwan
\textsuperscript{g} Sustainable Chemical Science and Technology, Taiwan International Graduate Program, Academia Sinica, Taipei, 11529, Taiwan

\begin{abstract}
Heat shock protein 90 (Hsp90) is a ubiquitous chaperone to interact with numerous proteins to regulate multiple cellular processes, especially during cell proliferation and cell cycle progression. Hsp90 exists in a high level in tumor cells and tissues, and thus serves as a prognostic biomarker or therapeutic target in cancers. We herein report that Hsp90 is subjected to S-glutathionylation, a redox-dependent modification to form a disulfide bond between the tripeptide glutathione and cysteine residues of proteins, primarily at C366 and C412 in the presence of reactive oxygen species. The modification led to the loss of the ATPase activity. The level of Hsp90 was obviously reduced by S-glutathionylation, owing to C-terminus of Hsc70-interacting protein (CHIP)-mediated ubiquitin proteasome system. S-glutathionylation of Hsp90 was found to cross-talk with its C-terminal phosphorylation of Hsp90 that impedes the binding of Hsp90 with CHIP, demonstrating the importance of chaperone code in modulating Hsp90 function. Further biophysical analyses indicated that S-glutathionylation caused structural change of Hsp90, underlying the aforementioned functional regulation. Moreover, in accordance with the analysis of 64 samples collected from patients of breast cancer, the expression level of Hsp90 inversely correlated with the glutathionylated status of Hsp90. The ratio of total expression to glutathionylated status of Hsp90 was coherent to expression of biomarkers in breast cancer sample, potentiating the prognostic value in the cancer treatment.
\end{abstract}

1. Introduction

Glutathione (GSH), the thiol-containing γGlu-Cys-Gly tripeptide, is mainly involved in the defense or rescue of various oxidative damage events \cite{ref1}, which represents a redox-dependent post-translational modification (PTM) to form a disulfide bond between GSH and cysteine residues of target proteins. S-glutathionylation affects protein stability, protein-protein interactions and ligand binding in response to various oxidative stimuli \cite{ref2}. Reactive oxygen species (ROS) are produced under oxidative stress to enhance the level of S-glutathionylation. Accumulating reports have indicated that glutathionylated proteins play a role in numerous cellular pathways, such as cell cycle, apoptosis, and inflammatory response, to influence diseases or cancer progression \cite{ref1}. For example, actin attenuates its affinity to interact with tropomyosin upon S-glutathionylation, leading to the reduced contractility of filaments during ischemia-reperfusion injury \cite{ref3}. Furthermore, mitochondrial thymidine kinase 2 is glutathionylated to decrease enzyme activity and induce its proteolytic degradation upon oxidative stress \cite{ref4}. Collectively these studies demonstrate S-glutathionylation to be a crucial regulator in controlling cellular functions and disease progression.

Heat shock protein Hsp90 (Hsp90) is an evolutionarily conserved and highly abundant protein accounting for 1–2% of total proteins in unstressed cells \cite{ref5}. Hsp90 interacts with more than 300 client proteins,
most of which are oncoproteins, to control several cellular functions including cell cycle, hormone signaling and stress regulation. In addition, Hsp90 is an ATP-dependent chaperone. Its ATPase activity is located in its N-terminus, in which ATP binding and the subsequent hydrolysis drive a protein conformational change for activation. The middle domain of Hsp90 is responsible for interacting with client proteins and co-chaperones. The conserved MEVED motif is located at the C-terminus of Hsp90 to associate with co-chaperones, such as C-terminus Hsc70-interacting protein (CHIP, a ubiquitin E3 ligase) or Hsp70-Hsp90-organizing protein (HOP) [6]. Furthermore, the chaperone function of Hsp90 is known to be regulated by several post-translational modifications, including phosphorylation, acetylation, oxidation and S-nitrosylation [6], explaining the importance of chaperone code of Hsp90 [7]. For example, phosphorylation of Hsp90 potentiates its interaction with apoptotic protease-activating factor 1 to abrogate cytochrome c-induced apoptosis [8]. S-Nitrosylation of Hsp90 was found to cause a conformational change and thus prevent protein dimerization, eradicating its protein chaperone function and the interaction with endothelial nitric oxide synthase [9,10]. Nevertheless, S-glutathionylation of Hsp90 still remains ambiguous. It is uncertain whether the modification occurs in the presence of ROS and how S-glutathionylation affects the function and/or activity of Hsp90.

Hsp90 expression is increased in cancer cells and tissues and thus plays a crucial role in maintaining the stability of several proteins in malignant transformation [11]. Inhibiting the ATPase activity of Hsp90 in tumor cells results in the dissociation and degradation of client proteins in cancers [12]. For example, the oncoprotein Bcr-Ab1 is susceptible to proteosomal degradation upon inhibition of Hsp90 ATPase activity, diminishing the proliferation of chronic myelogenous leukemia cells [13,14]. Furthermore, a high expression level of Hsp90 correlates with a low survival rate in breast cancer patients [15]. The inhibition of Hsp90 in breast cancer cells was shown to effectively abrogate breast cancer cell proliferation [15]. As a consequence, Hsp90 appears to be an attractive target of breast cancer for both clinical diagnosis and therapeutic intervention.

In this study, we demonstrated Hsp90 to be glutathionylated in vivo and in vitro, which facilitated its binding with CHIP and the corresponding degradation of Hsp90 via the ubiquitin proteasome-mediated pathway. Owing to Hsp90 S-glutathionylation, several client proteins involved in breast cancer proliferation became unstable and susceptible to ubiquitination-mediated degradation. Furthermore, we also identified a crosstalk between S-glutathionylation and phosphorylation in Hsp90. S-glutathionylation of Hsp90 impaired its C-terminal phosphorylation at S718 (equivalent to T725 and S726 in Hsp90β). Nevertheless, S-glutathionylation of Hsp90 still remains ambiguous. It is uncertain whether the modification occurs in the presence of ROS and how S-glutathionylation affects the function and/or activity of Hsp90.

2.1. Materials and methods

2.1. Reagents and antibodies

Hydrogen peroxide (H₂O₂), Dithiothreitol (DTT) and oxidized glutathione (GSSG) were purchased from Sigma. Cycloheximide (CHX) and MG-132 were from Calbiochem. Antibodies against GSH (D8) was from Virogen. Anti-Hsp90α (D-19), Hsp90α (F-8), CHIP (H-231), CK2 (C-18), and Raf-1 (C-20) antibodies were from Santa Cruz Biotechnology. Anti-GAPDH, PR, ER, and CDK4 were from Genetex. Anti-HER2 antibodies were from Cell signaling. Anti-Hsp90α (H90-10) antibody was from Enzo Life Sciences. pThr725/Ser726 Hsp90α antibody (GDD8.2) was kindly provided by Dr. Vojtesek at Masaryk Memorial Cancer Institute, Czech Republic.

2.2. Cell culture, transfection and plasmids

Human breast cancer cells MDA-MB-157, SKBR3 and T47D were purchased from American Type Culture Collection (ATCC). MDA-MB-157 cells were grown in MEM containing 10% fetal bovine serum (FBS). T47D cells were maintained in RPMI1640 medium containing 10% FBS. SKBR3 breast cancer cells were cultured in McCoy’s 5A medium supplemented 10% FBS. The full-length human HSP90β was subcloned into pcDNA3.1 and pET-15b vectors. The plasmids which mutation at cysteine 366, 412, 521 or 589 were generated by using QuickChange Site-Directed Mutagenesis Kit (Stratagene). For transfection, cells were transfected with indicated plasmid for by using Lipofectamine 2000 (Invitrogen). After transfection for 48 h, cells were treated with 1 mM H₂O₂ and harvested after 10 min. The total protein was extracted and subjected to immunoprecipitation or immunoblotting assay.

2.3. Immunoprecipitation and immunoblotting

For immunoprecipitation, breast cancer cells were treated with or without 1 mM H₂O₂ for 10 min, and lysed in lysis buffer (20 mM HEPES PH 7.4, 2 mM EDTA, 1% Triton X-100, 10% Glycerol, 1 mM DTT) containing protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor cocktail (Roche). Following removal of debris by centrifugation, protein concentrations of clarified lysates were determined by the BCA protein assay reagent kit (Thermo Fisher Scientific). The lysates containing equal amounts of proteins were precleared with protein A agarose (Thermo Fisher Scientific) and precipitated with anti-Hsp90β antibody (Santa Cruz Biotechnology or Enzo life Science). The precipitates were then mixed with 5X sample-loading buffer and analyzed by immunoblotting. The tumor samples from patients were finely minced and lysed in lysis buffer with homogenizer. After centrifugation, the total protein were extracted and immunoprecipitation was performed with Hsp90β antibody (Santa Cruz Biotechnology). After incubation with lysates from MDA-MB-157 cells, the recombinant 6xHis-tagged Hsp90β protein (His-Hsp90β) was precipitated with His Mag Sepharose Ni (GE Healthcare), and analyzed by immunoblotting. For immunoblotting assay, equal amounts of protein or precipitates were separated by Tris-glycine polyacrylamide gels, then transferred electro-photoretically onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were blocked and probed with the indicated antibodies.

2.4. Preparation of recombinant Hsp90β protein

Escherichia coli BL21 (DE3) cells were transformed with pET-15b-Hsp90β plasmids expressing the wild-type or mutant forms (C366A, C412A, C562A and C589A) of Hsp90β and cultured at 37 °C. Protein production was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 16 h. Recombinant proteins were then affinity purified by using Ni Sepharose affinity chromatography (GE Healthcare). Subsequently, His-Hsp90β was purified by ion-exchange chromatography with a HiTrap Q column (GE Healthcare). The protein was eluted at 1 M NaCl, 50 mM Tris-HCl, pH8.0. His-Hsp90β was further purified by a Superdex 200 size exclusion column (GE Healthcare). The fractions containing His-Hsp90β were collected and pooled. The purified proteins were stored in 50 mM Tris-HCl, 380 mM NaCl, 1 mM DTT, pH8.0.

2.5. ATPase activity assay

The ATPase activity of Hsp90 was measured using pyruvate kinase/
lactate dehydrogenase coupled assay [16]. The reaction was conducted in a final volume of 200 µl containing 2 mM ATP, 5 U lactate dehydrogenase (Sigma), 5 U pyruvate kinase (Sigma), 0.2 mM NADH, 1.5 mM phosphoenolpyruvate, 10 mM Tris-HCl, 20 mM KCl, 6 mM MgCl2, pH:7.5. Equal amounts of Hsp90β protein were used in each group. The decrease in NADH absorbance at 340 nm was recorded for 60 min using FlexStation 3 multi-mode microplate reader (Molecular Devices). Before the reaction, the NADH absorbance from the mixture without Hsp90β was recorded for 5 min as background of spontaneous ATP hydrolysis.

2.6. In vitro glutathionylation of recombinant Hsp90β protein

Recombinant Hsp90β (2 µg) was incubated with various concentrations of GSSG (0, 2, 5, and 10 mM) at 37 °C for 30 min. For demonstrating the formation of disulfide bond between glutathione and protein, the DTT was added to reaction for further 20 min. The glutathionylation level of Hsp90β protein was detected by 7.5% non-reducing SDS-PAGE and immunoblotting with anti-GSH antibodies.

2.7. Protein stability assay

MDA-MB-157 cells expressing wild-type or mutant forms of His-Hsp90β were treated with CHX in the presence or absence of GSSG for various intervals. The expression of His-Hsp90β was examined by immunoblotting with anti-His antibodies. For recombinant His-Hsp90β, proteins were treated with or without GSSG for 30 min and incubated with cell lysates from MDA-MB-157 cells for indicated times. The level of His-Hsp90β was detected using immunoblotting with anti-His antibodies.

2.8. Ubiquitination assay of Hsp90β protein

The His-Hsp90β protein was mixed with or without GSSG for 30 min, and subsequently incubated with cell lysate from MDA-MB-157 cells expressing HA-ubiquitin for further 30 min. The immunoprecipitation assay was performed with anti-His antibodies. The status of ubiquitination of His-Hsp90β protein was measured by immunoblotting with anti-HA antibodies.

2.9. CD spectroscopic measurement

After passing through the desalting column, the GSSG-treated or non-GSSG-treated His-Hsp90β protein was subjected to CD spectra analysis using a JASCO J-810 spectropolarimeter with thermostatically controlled at 37 °C. Far-UV CD spectra were recorded.

2.10. Patients and samples preparation

A cohort of 64 histologically confirmed breast cancer patients with complete follow-up protocols approved by the Institutional Review Board of Tri-Service General Hospital Hospital, Taipei, Taiwan, were enrolled in this study. Tumor samples were obtained during surgery and were fully encoded to protect patient confidentiality. The Institutional Review Board of Human Subjects Research Ethics Committees of Academia Sinica (Taipei, Taiwan) and Tri-Service General Hospital Hospital (Taipei, Taiwan) also approved this study.

3. Results

3.1. Hsp90 is glutathionylated in vitro and in intact cells

Our previous study indicated that Hsp90 is one of the proteins to be modified by S-glutathionylation under oxidative stress [17]. To verify the glutathionylation status of Hsp90 in cells, we performed an immunoprecipitation assay with specific antibodies and detected glutathionylation signals using a glutathione-specific antibody. As shown in Fig. 1A, Fig. S1 and Fig. S7A, the glutathionylation signals were strongly increased in breast cancer cells (MDA-MB-157 cells) that were exposed to higher oxidant concentrations. The level of PSSG-Hsp90β was quantified and normalized to His-precipitates (PSSG-Hsp90β) incubated with 2 mM GSSG by One-way ANOVA. All data are shown as the mean (±SD).
to oxidative stimuli (H$_2$O$_2$). When recombinant Hsp90 protein was incubated with oxidized glutathione (GSSG), the glutathionylation level was significantly increased in a dose-dependent manner (Fig. 1B and Fig. S7B). The signal was completely abolished by the addition of dithiothreitol (DTT), confirming that the modification was due to the formation of a disulfide bond between GSH and the cysteine residues of Hsp90 (Fig. 1B).

Previous study indicated that four cysteine residues (C366, C412, C521 and C589) of Hsp90β are involved in Hsp90β glutathionylation [17]. To identify the cysteine residues that are modified upon S-glutathionylation, each cysteine residue was replaced with alanine by site-directed mutagenesis (C366A, C412A, C521A and C589A). Further analysis showed that the glutathionylation signal of Hsp90β was significantly reduced in the mutant C366A- or C412A-transfected cells upon H$_2$O$_2$ treatment (Fig. 1C). Furthermore, in vitro observation showed that GSSG significantly induced glutathionylation of the Hsp90β protein, but the glutathionylation level was reduced more than 50% for the C366A and C412A mutant proteins, indicating that Cys366 and Cys412 are the primary residues participating in Hsp90β glutathionylation (Fig. 1D).

3.2. Glutathionylation inhibits the ATPase activity of Hsp90

Given that the ATPase activity of Hsp90 is essential for its activity in vivo [18], we tested whether glutathionylation could alter its ATPase activity. Notably, the ATPase activity of recombinant Hsp90β was reduced when Hsp90β was incubated with GSSG at various concentrations. Nevertheless, the reduced activity could be restored by addition of DTT (Fig. 2). Meanwhile, C366A, C412A and C366A/C412A mutant proteins were all found resistant to the GSSG treatment and therefore the ATPase activity was not decreased, unlike the wild type Hsp90β (Fig. S5). The result supported the idea that glutathionylation largely abrogated the ATPase activity of Hsp90β. We also examined the ATP-binding capacity of Hsp90β by detecting signal changes in fluorophore-labeled ATP (MANT-ATP). The fluorescent signal was significantly changed when Hsp90β protein treated with GSSG at the concentration of 10 mM, but not at 2 and 5 mM (Fig. S6A). The concentration-dependent change was likely due to the degree of glutathionylation. Additionally, the MANT-ATP binding was also examined by intrinsic fluorescence ($\lambda_{em} = 280$ nm). The emission of tryptophan fluorescence of Hsp90β showed a small decrease at the GSSG concentration of 2 and 5 mM, but a large reduction at 10 mM in association with a blue shift (~5 nm) in the $\lambda_{em}$ wavelength (Fig. S6B). The observed concentration dependence appeared to be consistent in both studies. Taken together, these results clearly demonstrated that GSSG-induced glutathionylation could greatly impair the ATPase activity of Hsp90β.

3.3. Oxidative stress-induced glutathionylation enhances Hsp90 degradation

The level of wild-type His-Hsp90β was dramatically decreased under oxidative stress, in sharp contrast to that of mutated His-Hsp90β (C366A or C412A) in transfected MDA-MB-157 cells when these cells were treated with H$_2$O$_2$ (Fig. 3A). We investigated whether glutathionylation plays a role in protein stability under H$_2$O$_2$–induced oxidative stress. To substantiate our findings in glutathionylation-mediated protein degrada- tion, cycloheximide (CHX) was used to blockade protein synthesis and study the protein turnover rate. As shown in Fig. 3B and C, the turnover rate of wild-type His-Hsp90β and the two mutants C521 and C589, was substantially faster after H$_2$O$_2$ treatment for 30 min as compared with the negative control (wild-type His-Hsp90β) without H$_2$O$_2$ exposure. Comparably, the cells expressing a mutation at C366 or C412 of His-Hsp90β displayed stable protein expression of His-Hsp90β after H$_2$O$_2$ treatment (Fig. 3B and C). The result indicated that oxidative stress-induced glutathionylation plays a key role in promoting protein degradation of Hsp90β.

3.4. Glutathionylation promotes Hsp90 degradation through the increased CHIP binding and the subsequent ubiquitination

The above-mentioned findings prompted us to investigate the molecular mechanism underlying glutathionylation-mediated regulation of protein stability. To delineate how glutathionylation facilitates Hsp90 degradation, purified His-Hsp90 was glutathionylated by treatment with GSSG (Hsp90β-GSSG) and subsequently incubated with cell lysates for different durations to assess protein stability. The level of His-Hsp90 protein was greatly decreased upon GSSG modification after the protein was incubated with cell lysate for 20 min and longer (Fig. 4A and Fig. S7C). Although the C366A and C412A mutants were also gradually degraded in the presence of GSSG, it appeared to be more resistant to protein degradation (Fig. 4A). Furthermore, the addition of a proteasome inhibitor (MG-132) completely inhibited the degradation of GSSG-treated Hsp90 (Fig. 4A and Fig. S7C), suggesting that glutathionylation-induced Hsp90 degradation is mediated by the proteasome system. The result was consistent with previous report that the degradation of Hsp90 was regulated by proteasome-mediated pathway [7].

Hsp90 protein degradation is controlled by the ubiquitin-proteasome system [7]. We next examined whether the S-glutathionylation of Hsp90 is connected with ubiquitination. When Hsp90β was treated with GSSG and the lysate of MDA-MB-157 cells, the level of ubiquitination in Hsp90β was significantly enhanced (Fig. 4B). In contrast, the ubiquitination level in the C366A and C412A mutants were significantly lower upon the treatment with GSSG (Fig. 4B), indicating a positive correlation between glutathionylation and ubiquitination of Hsp90β. Because CHIP is an E3 ligase specifically involved in the Hsp90β degradation [19], we investigated whether glutathionylation affects the binding between Hsp90 and CHIP. Treatment of Hsp90 protein with GSSG greatly

![Fig. 2. Glutathionylation inhibited the ATPase activity of Hsp90. Recombinant Hsp90β protein (2 μg) was incubated without or with oxidized glutathione (GSSG, at 2, 5 or 10 mM) for 30 min before an ATPase activity assay was performed. ATPase activity assay was conducted according to the protocol as described in materials and methods. The ATPase activity of Hsp90β protein without GSSG treatment was presented as 100%. ATPase activity of Hsp90β protein treated with GSSG was normalized to that of Hsp90β protein without GSSG treatment. *p < 0.05 versus Hsp90β protein without GSSG treatment. #p < 0.05, compared to Hsp90β protein with 10 mM GSSG stimulation by One-way ANOVA. Results are presented as the mean percentage (±SD) of three independent experiments.](image-url)
enhanced the binding with CHIP (Fig. 4C and Fig. S10A), whereas the mutation at C366 (C366A), C412 (C412A), C521A, and C589A) of Hsp90β resulted in a lower binding with CHIP (Fig. 4C). These observations supported the idea that glutathionylation enhances the binding of Hsp90 with CHIP, which leads to subsequent ubiquitination and proteasome-mediated protein degradation.

Moreover, we studied if the interaction between Hsp90 and its client proteins is regulated by the post-translational glutathionylation. Several Hsp90 client proteins are involved in breast cancer cell oncogenesis, including the receptor protein kinase erbB2 (HER2), estrogen receptor...
Fig. 4. S-glutathionylation promoted Hsp90 degradation through the increase of CHIP binding and ubiquitination. (A) Recombinant His-Hsp90β protein treated with or without 2 mM GSSG was incubated with cell extracts from MDA-MB-157 cells for the indicated times. MG-132 treatment (10 μM) was used to inhibit proteasome activity before His-Hsp90β protein exposure to GSSG. The expression level of His-Hsp90β protein was detected by western blot analysis with anti-His antibody and normalized to level of GAPDH. The relative expression of His-Hsp90β protein at 0 min was represented as 100% of protein level (n = 3). *p < 0.05, compared to that WT His-Hsp90β protein without incubation with GSSG. #p < 0.05, compared to WT His-Hsp90β protein incubated with GSSG by One-way ANOVA. (B, C) WT His-Hsp90β protein or His-Hsp90β protein mutated at C366 (C366A) or C412 (C412A) was treated with or without 2 mM GSSG for 30 min. After incubation with cell extracts from MDA-MB-157 cells expressing HA-ubiquitin for 30 min, an immunoprecipitation (IP) assay was performed using an anti-His antibody. The ubiquitinated His-Hsp90β protein was detected by western blotting analysis with anti-HA antibody. The ubiquitinated His-Hsp90β protein was quantified by normalization the density of a smear in each individual lane to the density of His-Hsp90β protein (ubiquitinated His-Hsp90β/His-Hsp90β) in the precipitate. After immunoprecipitation with anti-His antibody, the binding with CHIP was analyzed using western blot with anti-CHIP antibody and quantified by normalization to the level of His-Hsp90β protein in the precipitates (n = 3). *p < 0.05, versus wild-type Hsp90β protein without GSSG treatment. #p < 0.05, compared to wild-type Hsp90β protein treated with 2 mM GSSG by One-way ANOVA. Data are shown as the mean (±SD).
(ER), progesterone receptor (PR), as well as the downstream signaling proteins of HER2 (such as Raf-1 and CDK4). Their stability was regulated via their protein-protein interaction with Hsp90 [20]. The interaction of Hsp90β with HER2, ER, PR, Raf-1, and CDK4 was decreased in the presence of GSSG (Fig. S8), supporting the idea that Hsp90 glutathionylation indeed impaired the binding of Hsp90 with its client proteins in cancer cells.

3.5. Hsp90 glutathionylation diminishes its C-terminal phosphorylation mediated by CK2 to affect CHIP binding

The binding between Hsp90 and CHIP can be prevented by phosphorylation on its C-terminal region primarily mediated by casein kinase 2 (CK2). The phosphorylated sites at S718 of Hsp90β and T725 and S726 of Hsp90α were previously shown to reduce the binding of CHIP to Hsp90 [21]. We prepared the S718D mutant of Hsp90β serving as the mimic to the phosphorylated S718 of Hsp90β. The S718D mutant displayed reduced binding with CHIP upon GSSG-induced glutathionylation, as compared to the wild type Hsp90β (Fig. 5). Likewise, the phosphorylated level of T725 and S726 (pThr725/Ser726) in Hsp90α was also decreased after the protein was treated with GSSG (Fig. S10A). Both results demonstrated that the glutathionylation of Hsp90 can interfere in its C-terminal phosphorylation (catalyzed by CK2) and thus promote its binding with CHIP for the subsequent protein degradation.

3.6. S-glutathionylation induces a conformational change in Hsp90

To evaluate whether glutathionylation could impact the Hsp90 structure, purified Hsp90β proteins was treated with various concentrations of GSSG, followed by examination with circular dichroism (CD) analysis. Hsp90β displayed major absorption in 200 and 203 nm in the CD spectrum, in consistence with a high content of α-helixes (42.7%, see Table 1 and Fig. 6). GSSG treatment induced secondary structure alteration of Hsp90β in a dose-dependent manner. Comparing the CD

![Fig. 5. Hsp90 S-glutathionylation impaired CK2 recruitment and the subsequent C-terminal phosphorylation, leading to the increased CHIP binding. (A) Purified WT His-Hsp90β protein or His-Hsp90β mutated at S718 (S718D) was treated with or without 2 mM GSSG and then mixed with cell lysates from MDA-MB-157 cells for 30 min. Subsequent immunoprecipitation was performed with an anti-His antibody. Immunoprecipitated proteins were analyzed by western blot analysis with anti-CHIP antibody. The binding of CHIP to His-Hsp90β protein was measured by the expression of CHIP to the level of His-Hsp90β in the precipitates (n = 4). *p < 0.05, compared to WT His-Hsp90β protein without GSSG stimulation. "p < 0.05, compared to wild-type His-Hsp90β protein treated with 2 mM GSSG by One-way ANOVA. (B) His-Hsp90β protein incubated with 2 mM GSSG for 30 min was mixed with total cell protein from MDA-MB-157 cells for 30 min. Immunoprecipitation assay was performed by anti-His antibody. The interaction of CK2 and His-Hsp90β protein was further analyzed in the precipitates by western blot using anti-CK2 antibody. The binding of CK2 to His-Hsp90β protein was analyzed by the ratio of CK2/His-Hsp90β protein (n = 4). *p < 0.05, compared to His-Hsp90β protein without GSSG incubation. Results are presented as the mean percentage (±SD).]
Fig. 6. The structure of Hsp90 was altered after S-glutathionylation. Recombinant His-Hsp90β protein (2 μg) was incubated without or with various concentrations of GSSG for 30 min. Far-UV CD spectra were measured to analyze the changes in secondary structures of His-Hsp90β protein.

spectra of Hsp90β with and without GSSG treatment indicated that GSSG-induced glutathionylation caused 5.7% decrease in the α-helix content, accompanied by 5% increase in others. Additionally, the same sample was also subjected to the aforementioned measurement of intrinsic fluorescence. In the presence of MANT-ATP, the emission intensity of tryptophan fluorescence showed a large decrease upon GSSG-induced glutathionylation (treatment with GSSG of 10 mM), in company with a 5-nm blue shift in the λem wavelength (Fig. S6B). The results implicated that S-glutathionylation caused conformational change and the resulting Hsp90β became less ordered and compact.

3.7. Inverse correlation between glutathionylation and protein expression levels of Hsp90 is evident in patients with varying breast cancer subtypes

An elevated level of Hsp90 has been associated with malignant breast cancer progression and decreased survival in primary breast cancer patients [22]. Our current findings indicated that Hsp90 glutathionylation promoted its degradation. Therefore, we examined the correlation between the expression level and glutathionylated status of Hsp90β in the clinical specimens collected from patients of breast cancer. High expression levels of total Hsp90β were associated with low levels of glutathionylated Hsp90β, revealing a negative correlation (R = 0.5095) on the basis of linear regression analysis (Fig. 7A and B, and Fig. S11). It potentiates the clinical significance of glutathionylated Hsp90β in the diagnosis or prognosis of breast cancer. Additionally, the ratio of total expression to glutathionylated Hsp90β was increased in breast cancer patients with HER2 overexpression but without ER and PR expression, providing a prognostic value in breast cancer (Fig. 7C-E).

4. Discussion

Herein we identified S-glutathionylation of Hsp90 that represents another cysteine modification in Hsp90, in addition to oxidation and S-nitrosylation [23]. S-Nitrosylation of C597 in Hsp90 was previously reported to affect its dimerization and thus cause the loss of the ATPase activity [9]. Interestingly, in this study S-glutathionylation preferentially occurs at C366 and C412 that are located in the middle domain of Hsp90β (Fig. S12), in company with the abolished ATPase activity (Fig. 2). Double mutations at sites of C366 and C412 displayed a similar effect on the degree of glutathionylation and the ATPase activity (Figs. S4 and S5, respectively). Further examination by CD and intrinsic fluorescence analyses revealed that glutathionylation led to structural changes of Hsp90 (Fig. 6, Table 1 and Fig. S6). The resulting conformational change likely impeded the binding of Hsp90 with its client proteins (Fig. S8).

Moreover, several reports showed that Hsp90 can be cleaved in different ways under oxidative stress. For example, somatostatin treatment inhibits the production of a small, approximately 70-kDa fragment of Hsp90 via abolishing calpain cleavage in mouse monocytes [24]. UVB irradiation promotes Hsp90β cleavage near its N-terminus to produce a 55-kDa fragment in human epidermoid carcinoma cells [25]. In addition, ascorbate/menadione induces oxidative stress in a human erythroleukemic cell line, resulting in the cleavage in the N-terminal region of Hsp90 to produce two fragments of 72 and 18 kDa [26]. In contrast, our results showed that the oxidative stress-induced glutathionylation enhanced Hsp90 degradation by promoting the ubiquitin-proteasome pathway in breast cancer cells. However, we did not detect any fragment of Hsp90 after glutathionylation by using the antibody specific for the C-terminal region (amino acids 610-723) of Hsp90 (Fig. S1). These dissimilar scenarios might be reflected by different regulations on the Hsp90 protein level upon various stimuli of ROS.

S-glutathionylation has been shown to crosstalk with other PTMs to modify proteins that control cellular signaling events [27]. The interplay between S-glutathionylation and phosphorylation is involved in modulating functions of the cardiovascular system. Vascular smooth muscle cells treated with angiotensin II result in S-glutathionylation of Ras protein and the subsequent phosphorylation of p38 and Akt, contributing to vascular hypertrophy [28]. S-glutathionylation of the STAT3 transcription factor attenuates its phosphorylation at Y705 and affects the downstream signaling pathway [29]. Furthermore, S-glutathionylation may regulate the processes of ubiquitination and sumoylation though modulating the interaction of E3 ligase with several protein targets [30,31]. These evidence indicated that the function of proteins is deliberately regulated between S-glutathionylation and other PTMs. We herein discovered another interplay among S-glutathionylation, phosphorylation and ubiquitination in Hsp90 to decipher how the Hsp90 chaperone code affects its functions. The phosphorylation of Hsp90α at T725 and S726, and Hsp90β at S718 was mainly mediated by CK2, which prevents Hsp90 from binding with CHIP to proceed towards protein degradation [21]. Although CK2, casein kinase 1 (CK1), and glycogen synthase kinase 3beta (GSK3β) mainly target C-terminus of Hsp90, only the binding of CK2 to Hsp90 was inhibited after GSSG-mediated glutathionylation (Fig. 5B, Fig. S10B and Fig. S9). In particular, S-glutathionylation diminished the phosphorylation of Hsp90α at T725 and S726, which facilitates CHIP-mediated protein ubiquitination and subsequent proteosomal degradation (Fig. S10). Compared to WT Hsp90β protein, phospho-mimetic Hsp90β protein (S718D) diminished its binding with CHIP, further strengthening the crosstalk of S-glutathionylation, phosphorylation, and ubiquitination in Hsp90 (Fig. 5A).

CK2 was reported to phosphorylate Thr22 in Hsp90α to interrupt the hydrophobic interaction between the N-terminal domain and middle domain of Hsp90α, resulting in aberrant chaperone function [32]. We identified another interdomain communication in Hsp90α where the glutathionylation at middle domain (C366 and C412) not only promoted the CK2 access from phosphorylating C-domain of Hsp90β (S718), and thus enhanced CHIP binding and the subsequent ubiquitination.

Hsp90 is highly expressed in malignant tumors and is associated with a decreased survival rate in breast cancer patients [15,33]; Hsp90 is therefore an attractive target in cancer therapy. Several oncoproteins involved in breast cancer growth and survival, including HER2, AKT, ER, and PR, are client proteins of Hsp90 [20]. Hsp90 degradation was enhanced by S-glutathionylation, and the chaperone function of Hsp90

| Table 1 | Secondary structure content of recombinant Hsp90β in non-glutathionylated or glutathionylated forms. |
|---------|-------------------------------------------------|
|         | α-helix | β-sheet | Turn | Others |
| Hsp90β | 42.7% | 17.0% | 12.4% | 27.9% |
| Hsp90β-SSG | 37.0% | 17.2% | 11.9% | 33.9% |
to interact with client proteins was significantly decreased (Fig. S8).
Clinical data demonstrated that an inverse correlation between the levels and glutathionylation status of Hsp90, and the ratio of expression to glutathionylated level of Hsp90 was correlated with ER, PR, and HER2 expression in tumors from breast cancer patients (Fig. 7). Therefore, the ratio of total expression to glutathionylated status of Hsp90 can be considered as a prognostic indicator during the therapeutic treatment of breast cancer. In general, the ratio of GSH/GSSG is about 100:1 under physiological condition [34]. Decreased level of GSH in cancer cells results is often associated with defective response to oxidative stress [35]. Since S-glutathionylation is a reversible modification on cysteine residues of proteins, evaluating the levels of blood GSH and GSH/GSSG could well reflect the status of glutathionylated Hsp90 in tumor tissues.

In conclusion, we demonstrated that Hsp90 can be glutathionylated and subsequently ubiquitinated and degraded by the ubiquitin-proteasome system to lose its chaperone function. Clinical results revealed that the inverse correlation between total expression level of Hsp90 and the glutathionylated status, in which the ratio was also correlated with the level of biomarkers in breast cancer samples to as a prognostic marker. Therefore, future studies should use these results to define the level of glutathionylated Hsp90 as a more effective strategy to inhibit Hsp90 function. We suggest that Hsp90 glutathionylation is a promising therapeutic strategy for breast cancer.

**Funding**

The study of this manuscript was financially supported by Academia Sinica, Chinese Medical University Hospital (DMR-111-180) and National Science and Technology Council (MOST 110-2314-B-039-057- and 111-2314-B-039-049-).

**Acknowledgement**

We thank Dr. Meng-Ru Ho at the Biophysics Core Facility, Academic Sinica for his technical assistance.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102501.
References

[1] A. Pastore, F. Piemonte, S-Glutathionylation signaling in cell biology: progress and prospects, Eur. J. Pharmacuet. Sci. 5 (official journal of the European Federation for Pharmaceutical Sciences 46 (2012) 279–292.

[2] I. Dalle-Donne, R. Rossi, G. Colombo, D. Giustarini, A. Milzani, Protein S-glutathionylation: a regulatory device from bacteria to humans, Trends Biochem. Sci. 34 (2009) 85–96.

[3] F.C. Chen, O. Ogut, Decline of contractility during ischemia-reperfusion injury: act glutathionylation and its effect on allosteric interaction with tropomyosin, Am. J. Physiol. Cell Physiol. 290 (2006) C719–C727.

[4] S. Sun, S. Eriksson, L. Wang, Oxidative stress induced S-glutathionylation and proteolytic degradation of mitochondrial thymidine kinase 2, J. Biol. Chem. 287 (2012) 24304–24312.

[5] B.T. Lai, N.W. Chin, A.E. Stanek, W. Keh, K.W. Lanks, Quantitation and intracellular localization of the Bsk heat shock protein by using monoclonal and polyclonal antibodies, Mol. Cell. Biol. 4 (1984) 2802–2810.

[6] A.G. Erlejman, M. Lagadari, J. Tonoce, G. Pivienn-Pillpuk, M.D. Galignana, Regulatory role of the 90-kDa-heat-shock protein (Hsp90) and associated factors on gene expression, Biochim. Biophys. Biophys. Acta 1839 (2014) 71–87.

[7] S.J. Backe, R.A. Sager, M.R. Woodford, A.M. Makedon, M. Mollapour, Post-translational modifications of Hsp90 and translating the chaperone code, J. Biol. Chem. 295 (2020) 11099–11117.

[8] M. Kurokawa, C. Zhao, T. Reyna, S. Kombluh, Inhibition of apoptosis formation by suppression of Hsp90beta phosphorylation in tyrosine kinase-induced leukemias, Mol. Cell. Biol. 28 (2008) 5494–5506.

[9] A. Martinez-Ruiz, L. Villanueva, C. Gonzalez de Orduna, D. Lopez-Ferrer, A. Higueras, C. Tarin, I. Rodriguez-Crespo, J. Vazquez, S. Lamas, S-nitrosylation of gene expression, Biochim. Biophys. Acta 1839 (2014) 71.

[10] P. Connell, C.A. Ballinger, J. Jiang, Y. Wu, L.J. Thompson, J. Hohfeld, C. Patterson, A. Martinez-Ruiz, L. Villanueva, C. Gonzalez de Orduna, D. Lopez-Ferrer, A. Higueras, C. Tarin, I. Rodriguez-Crespo, J. Vazquez, S. Lamas, S-nitrosylation of gene expression, Biochim. Biophys. Acta 1839 (2014) 71.

[11] A. Pastore, F. Piemonte, Protein glutathionylation in cardiovascular diseases, Int. J. Mol. Sci. 14 (2013) 20845–20876.

[12] E. Buttirini, E. Darra, G. Chiavagato, B. Cellini, F. Cozolino, M. Monti, P. Pucci, D. Dell’Orco, S. Mariotto, S-Glutathionylation at Cys528 and Cys542 impairs STAT3 phosphorylation, ACS Chem. Biol. 9 (2014) 1885–1893.

[13] Y. Watanabe, C.E. Murdoch, S. Sano, Y. Ido, M.M. Bachschmid, R.A. Cohen, R. Matsui, Glutathione adducts induced by ischemia and deletion of glutaredoxin-1 stabilize HIF-1alpha and improve limb revascularization, Proc. Natl. Acad. Sci. U.S.A. 113 (2016) 6011–6016.

[14] Y. Watanabe, K. Watanabe, D. Fujioka, K. Nakamura, T. Nakamura, M. Uematsu, M.M. Bachschmid, R. Matsui, K. Kugiyama, Protein S-glutathionylation stimulate adipogenesis by stabilizing C/EBPbeta in 3T3L1 cells, Faseb. J. 34 (2020) 5827–5837.

[15] M. Mollapour, S. Tsutsumi, A.W. Truman, W. Xu, K.D. Vaughan, K. Beebe, A. Konstantinova, S. Vourganti, B. Panaretou, P.W. Piper, J.B. Trepel, C. Prodromou, L.H. Pearl, L. Neckers, Threonine 22 phosphorylation attenuates Hsp90 interaction with cochaperones and affects its chaperone activity, Mol. Cell. 41 (2011) 672–681.

[16] M.E. Gorre, K. Ellwood-Yen, G. Chiosis, N. Rosen, C.L. Sawyers, BCR-ABL point mutations isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90, Blood 100 (2002) 3041–3044.

[17] E. Pick, Y. Kluger, J.M. Gilmane, C. Moeder, R.L. Camp, D.L. Rimn, H.M. Kluger, High HSP90 expression is associated with decreased survival in breast cancer, Cancer Res. 67 (2007) 2922–2927.

[18] J.A. Ali, A.P. Jackson, A.J. Howells, A. Maxwell, The 43-kilodalton N-terminal fragment of the DNA gyrase B protein hydrolyzes ATP and binds coumarin drugs, Biochemistry 32 (1993) 2717–2724.

[19] B.Y. Chiang, C.C. Chou, F.T. Hsieh, S. Gao, J.C. Lin, S.H. Lin, T.C. Chen, K.H. Kho, C.H. Lin, In vivo tagging and characterization of S-glutathionylated proteins by a chemoenzymatic method, Angew. Chem. 51 (2012) 5871–5875.