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Hsp90 inhibition renders iNOS aggregation and the clearance of iNOS aggregates by proteasomes requires SPSB2

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ABSTRACT

Inducible nitric oxide synthase (iNOS) plays important roles in cell injury and host defense. Our early study demonstrated that heat shock protein 90 (Hsp90) interacts with iNOS and this interaction enhances iNOS function. Recently, we reported that Hsp90 is also essential for iNOS gene transactivation. In the present study, we investigate the role of Hsp90 in controlling iNOS protein stability. In mouse macrophages, Hsp90 inhibition dissociated Hsp90 from iNOS and the latter subsequently formed aggregates. Aggregation deactivated iNOS. iNOS aggregates were cleared by the ubiquitin-proteasome system (UPS) inside cells. CHIP, an Hsp90-dependent E3 ligase, was previously implicated in iNOS turnover. However, CHIP knockdown had little effect on iNOS degradation in Hsp90-inhibited cells, indicating that other E3 ligases accounted for the clearance of iNOS aggregates. Further studies revealed that the SPRY domain-containing SOCS box protein 2 (SPSB2), an E3 ligase-recruiting protein, was essential for the ubiquitination of iNOS aggregates. SPSB2 knockdown or deleting the SPSB2-interacting domain on iNOS prevented the clearance of iNOS aggregates in Hsp90-inhibited cells. Thus, besides modulating iNOS function and gene transcription, Hsp90 is also essential for the protein stability of iNOS. Hsp90 blockade induces iNOS aggregation and SPSB2 is required for UPS degradation of iNOS aggregates.

1. Introduction

Nitric oxide (NO) is a gaseous free radical. Although physiological concentrations of NO serve as a signaling molecule in neuronal transmission and cardiovascular regulation, high levels of NO harm cells [1–4]. Hence NO is employed by the immune system in the fight against microbe invaders and tumor cells [5–7]. In mammals, NO is derived from L-arginine via a reaction catalyzed by a family of NO synthase (NOS) including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [7–9]. Among them, iNOS participates in cell injury and host defense [10,11]. In contrast to the constitutively existing nNOS and eNOS, little iNOS can be detected in normal cells and tissues. Inflammatory mediators, such as bacterial product lipopolysaccharide (LPS) and cytokines including interferon-γ (IFN-γ), are potent inducers of iNOS gene expression. All NOS isoforms are activated by their binding with the cofactor calmodulin (CaM) [8,12,13]. For nNOS and eNOS, the binding to CaM is facilitated by the rise of intracellular Ca^{2+}. iNOS, however, contains an intrinsically bound CaM and stays constantly active once expressed [8,12,13]. The continuous activity along with the high NO-generating efficacy is thought to suit iNOS for its functions in host defense and cell injury.

Because iNOS is constantly active, the regulation of NO production from iNOS was thought to primarily occur at the enzyme transcriptional level. This notion evolves as recent studies show that iNOS function can be affected by protein-protein interactions and posttranslational modifications [14]. In an early study, we showed that iNOS associates with heat shock protein 90 (Hsp90) in cells and this association enhances iNOS activity [14]. The interaction with Hsp90 is found to be important in the cytotoxic effect of iNOS on cells. Recently, we reported that Hsp90 is necessary for the transcriptional activation of iNOS gene in cells stimulated by both LPS and IFN-γ [15]. Hsp90 appeared to be essential for transcriptional factor NF-κB and STAT1 to bind with the iNOS promoter during gene transactivation. The necessary role of Hsp90 in iNOS induction was confirmed in vivo in myocardium infarction [15]. Together, these studies demonstrate the importance of Hsp90 in regulating iNOS function and gene expression.

In addition to gene expression, the levels of active iNOS in cells are also determined by its protein stability and turnover [16–18]. Whether

Abbreviations: Hsp90, heat shock protein 90; NO, nitric oxide; iNOS, inducible NO synthase; SPSB2, SPRY domain-containing SOCS box protein 2; CHIP, carboxyl terminus of Hsc70 interacting protein; UPS, ubiquitin-proteasome system; GA, geldanamycin

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or not Hsp90 affects iNOS protein stability, and if it does, how changed iNOS stability is coped with inside cells are the remaining questions in the study of Hsp90 regulation of iNOS. In the present study, we address these issues in mouse macrophages which are stimulated to express iNOS. Our studies find Hsp90 vital for iNOS protein stability. Loss of the interaction with Hsp90 leads to iNOS aggregation and deactivation. Cells employ the ubiquitin-proteasome system (UPS) to eliminate aggregated iNOS proteins. We further reveal that the SPRY domain-containing SOCS box protein 2 (SPSB2), an E3 ligase-recruiting protein, is essential for the proteasomal clearance of iNOS aggregates in cells.

2. Materials and methods

2.1. Materials

Cell culture materials were purchased from Invitrogen (Carlsbad, CA). The antibody against iNOS was from BD Transduction Laboratories. Antibody against Hsp90 was a product of Cell Signaling Technology (Beverly, MA). The antibody against SPSB2 was from Santa Cruz Biotechnology (Santa Cruz, CA). LPS, recombinant mouse IFN-γ, geldanamycin, radicicol, anti-GAPDH and anti-flag antibodies were products of Sigma (St. Louis, MO). Unless otherwise indicated, all other chemicals used in this study were from Sigma.

2.2. Cell culture

Mouse macrophage (RAW 264.7, ATCC), human embryonic kidney 293 (HEK293), and African green monkey SV40-transfected kidney fibroblast (COS-7) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum in a 37 °C humidified atmosphere of 95% air and 5% CO_2. Expression of iNOS in RAW 264.7 cells was induced by LPS (2 μg/ml, serotype 026:B6) and IFN-γ (100 U/ml).

2.3. shRNA

HuSH 29mer shRNA constructs against CHIP gene (Origene Technologies) were transfected into HEK293 cells by using Lipofectamine 2000 reagents (Invitrogen). The CHIP knockdown efficiency was confirmed by Western blotting and the CHIP-depleted cells were subjected to further treatments and analyses.

2.4. siRNA

Small interfering RNA (siRNA) oligonucleotides targeting SPSB2 and control nonspecific siRNA were purchased from Santa Cruz Biotechnology. In twelve-well plates, cells were seeded the day before transfection and grown to 30% confluence. siRNA oligonucleotides (100 nM) were transfected into cells by using Lipofectamine 2000 reagents. After 48 h of transfection, cells were subjected to further experiments.

2.5. Plasmid construction

To construct the plasmid encoding 50–1144 truncated iNOS, the 50–1144 region of iNOS was PCR-amplified from previously constructed pCMV-iNOS plasmid using primers 5'-CCCAAGCTTGGGATGG GCTCCCCGCAGC and 5'-CCGCTCGAGCGGGCCAGAAGCTGGAAC. After overnight incubation with *Hin*dIII and *Xho*I, 50–1144 iNOS cDNA was cloned into the mammalian expression vector pCMV-Flag-Tag2B using the standard molecular biology procedures. To construct pEGFP-C3/iNOS plasmid encoding GFP-iNOS fusion protein, the *Hin*dIII-*Xho*I fragment of pCMV-iNOS plasmid containing iNOS cDNA was cloned into *Hin*dIII-*Sal*I sites of pEGFP-C3 vector.

2.6. Cell fractionation

Cells were rinsed with phosphate-buffered saline and lysed on ice for 30 min in a lysis buffer containing moderate detergents (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, 1 mM EDTA and protease inhibitor tablet). After a centrifugation at 14,000*g* for 15 min at 4 °C, the supernatants and pellets were recovered as soluble and insoluble fractions, respectively. The insoluble pellets were washed by PBS, and boiled in 1.5 × SDS/PAGE sample buffer (90 mM Tris-HCl, pH 6.8, 3% SDS, 15% glycerol, 0.01% Bromophenol blue and 62.5 mM dithiothreitol) for 7 min. Total cell samples were obtained by passing the lysates through 30 G needles after 30 min incubation in high-detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% SDS, 1 mM EDTA and protease inhibitor tablet).

2.7. Western blotting

Cells were harvested and lysed on ice for 30 min in lysis buffer, followed by 15 min centrifugation at 14,000*g*. Protein concentrations were determined by using the detergent-compatible protein assay kit (Bio-Rad). After 5 min boiling in the SDS/PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 40 mM dithiothreitol, 10% glycerol, and 0.01% Bromophenol blue), the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected with secondary antibodies conjugated with horseradish peroxidase. Immunoblots were developed on films using the enhanced chemiluminescence technique (SuperSignal West Pico, Pierce).

2.8. Fluorescence imaging

Plasmids encoding GFP-iNOS fusion proteins were transfected into HEK293 or COS-7 cells with Lipofectamine 2000 reagents. After 20 h of plasmid transfection, the cells were treated with MG-132 (20 μM) for 30 min prior to 8 h incubation with geldanamycin (GA, 5 μM) or radicicol (20 μM). Imaging was performed on a fluorescence microscope (Nikon Eclipse 80i) with the Qimaging Retiga-2000R digital camera (Qimaging).

2.9. iNOS activity assay

iNOS activity was measured by the L-[^{14}C]arginine to L-[^{14}C]citrulline conversion assay [19]. iNOS expression was induced in RAW 264.7 by LPS/IFN-γ for 12 h. Cells were then homogenated in homogenate buffer (50 mM Tris-HCl, pH7.4, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture). After centrifugation (14,000*g* for 30 min at 4 °C), the supernatants were harvested and used for measuring soluble iNOS activity. To obtain aggregated iNOS, LPS/IFN-γ-stimulated cells were incubated with GA (5 μM) or radicicol (20 μM) in the presence of MG-132 (20 μM) for 18 h. The cells were then harvested and homogenated in homogenate buffer. After centrifugation and stringent wash, the pellets were resuspended in the homogenate buffer and used for activity measurements of aggregated iNOS. The cell lysates were added to the reaction mixture containing 50 mM Tris-HCl, pH 7.4, 0.5 mM NADPH, 10 nM $CaCl_2$, 10 μg/ml CaM, 10 μM BH_4, 2 μM L-[^{14}C]arginine, and 36 μM L-arginine. After 15 min incubation at 37 °C, the reactions were terminated by ice-cold stop buffer. L-[^{14}C]Citrulline was separated by passing the reaction mixture through Dowex AG 50W-X8 (Na^+ form; Sigma) cation exchange columns and quantitated by liquid scintillation counting.

2.10. Nitrite assay

Total nitrite released in cell culture medium was measured with a