CHIP facilitates ubiquitination of inducible nitric oxide synthase and promotes its proteasomal degradation

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ABSTRACT

Inducible nitric oxide synthase (iNOS) is responsible for nitric oxide (NO) synthesis from L-arginine in response to inflammatory mediators. It is reported that iNOS is degraded mainly by the ubiquitin–proteasome pathway in RAW264.7 cells and human embryonic kidney (HEK) 293 cells. In this study, we showed that iNOS was ubiquitinated and degraded dependent on CHIP (COOH terminus of heat shock protein 70-interacting protein), a chaperone-dependent ubiquitin ligase. The results from overexpression and RNAi experiments demonstrated that CHIP decreased the protein level of iNOS, shortened the half-life of iNOS and attenuated the production of NO. Furthermore, CHIP promoted ubiquitination and proteasomal degradation of iNOS by associating with iNOS. These results suggest that CHIP plays an important role in regulation iNOS activity.

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1. Introduction

Nitric oxide, a pleiotropic regulator, is involved in many biological processes, including immune function, vasodilatation, synaptic transmission, and memory [1,2]. Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS). There are three types of NOS: inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) [3,4]. All NOS isoforms have similar primary structures, including an oxygenase domain at the N-terminus, a reductase domain at the C-terminus, a hinge calmodulin domain in between [1]. eNOS and nNOS are constitutively expressed, which are Ca2+-dependent and generate low levels of NO [5]. In contrast, iNOS produces a relative large amount of NO and its catalytic activity is Ca2+-independent. A number of agents, such as lipopolysaccharides and inflammatory cytokines, lead to iNOS expression in many human cells including macrophages, hepatocytes, chondrocytes, cardiac myocytes, and a variety of cancer cells [5]. High level of NO generated by iNOS has been proved to be linked with inflammation, neurodegenerative diseases, cardiovascular diseases, and cancer initiation, progression and promotion [6–8]. Therefore, iNOS has been considered to be a potential target for molecule-based disease therapy.

The activity of an enzyme can be controlled through the regulation of its synthesis, catalytic activity, or degradation. For iNOS, much is known about factors affecting its synthesis and catalytic activity, while it is not clear about the regulation of its degradation. Musial and Eissa have reported that the 26S proteasome is the major pathway responsible for degradation of iNOS in HEK 293 cells and RAW264.7 cells [6]. Further studies indicate that iNOS is subject to ubiquitination and that ubiquitination is required for its degradation in HEK293 cells and RAW264.7 cells [5,10]. However, the detailed mechanisms including the effects of specific regulatory factors on iNOS ubiquitination and proteasomal degradation remain to be elucidated.

CHIP, carboxyl terminus of HSC70-interacting protein, is a dimeric 35 kDa cytoplasmic protein whose amino acid sequence is highly conserved across species. The molecule of CHIP is characterized by three functional domains: a U-box domain at the C-terminus, a tetratricopeptide (TPR) domain at the N-terminus and a hinge calmodulin domain in between [1]. CHIP acts with chaperones HSC/HSP70 and HSP90 through its TPR domain. Additional chaperone client proteins, such as nNOS, Smad3, ErB2, tau and cystic-fibrosis transmembrane-conductance regulator (CFTR) [15–19]. Additionally, CHIP regulates eNOS trafficking through the Golgi compartment by a co-chaperone-dependent mechanism [20]. Although Jiang et al. showed that CHIP decreased iNOS protein level when CHIP and iNOS were co-transfected into COS cells [20], it is unknown how CHIP down-regulates iNOS and whether or not it is involved in ubiquitination and degradation of iNOS.
In the current study, we found that CHIP interacted with iNOS, shortened the half-life of iNOS by promoting ubiquitination and degradation of iNOS, and as a result, inhibited NO production.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal antibody against CHIP was from MERK. Monoclonal antibody against Myc-tag was from Roche Applied Science. Monoclonal antibody against HA-tag and Flag-tag were from Sigma. The anti-iNOS monoclonal antibody was obtained from BD PharMingen. Antibodies to ubiquitin and protein A/G were from Santa Cruz Biotechnology. Secondary antibodies coupled to IRDye 800 fluorophore for use with the Odyssey Infrared Imaging System were purchased from Rockland. LPS (from Escherichia coli 0111: B4) and N-benzoyloxycarbonyl(Z)-Leu-Leu-Leucinal (MG-132) were from Sigma. Dimethyl sulfoxide (Me2SO/DMSO) was from Amersco (USA). Cycloheximide (CHX) was obtained from Calbiochem.

2.2. Plasmid constructs

pcDNA3-HA-Ubiquitin was a generous gift from Dr. Ze’ev Ronai (The Burnham Institute, USA) [21,22]. pCMV-Myc-CHIP, pCMV-HA-CHIP and pBS-U6-CHIP were kindly provided by Dr. Zhijie Chang (Department of Biological Sciences and Biotechnology and School of Medicine, Tsinghua University, PR China) and experiment was carried out to knockdown endogenous CHIP successfully as described previously [23]. pcDNA3-iNOS was a generous gift form Dr. Solomon H. Snyder (The Johns Hopkins University School of Medicine, USA) [24]. The pcDNA3.1-Myc-iNOS was sub-cloned into pcDNA3.1. All expression vectors were sequenced to confirm.

2.3. Cell culture and transfection

The murine macrophage-like RAW264.7 cells and human embryonic kidney (HEK) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO2. Transient transfection was performed with a modified calcium phosphate method or by the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. In all cases, the total amount of DNA was normalized by the empty control plasmids.

2.4. Immunoprecipitation and Western blotting analysis

Cell lysates were centrifuged (15,000 g) at 4 °C for 15 min. Proteins were immunoprecipitated with indicated antibodies, respectively. The precleared Protein A/G PLUS–agarose beads (Santa Cruz Biotechnology) were incubated with immunocomplexes for 2 h and washed four times with the lysis buffer. The immunoprecipitates were subjected to SDS–PAGE followed by transferring onto polyvinylidene difluoride (PVDF) (Roche Applied Science) or nitrocellulose membrane (Hybond-C, Amersham Biosciences). The Western blot analyses were performed as described previously [25]. The antibody–antigen complexes were visualized by the LI-COR Odyssey Imaging System according to the manufacturer’s instruction using IRDye800 fluorophore-conjugated antibody (LI-COR Biosciences, Lincoln, NE) or using TMB Western blot system (Promega, Madison, WI, USA). Quantification was directly performed on the blot using the LI-COR Odyssey Analysis software.

2.5. Nitrite analysis

NO synthesis was spectrophotometrically determined by assaying the culture supernatants for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard.

2.6. Statistics

All experimental data obtained from cultured cells were expressed as mean ± SD. Western blotting analysis experiments were repeated 2–3 times with similar trends. A one-way repeated measure analysis of variance and a Student’s t-test were used to determine the significance of the difference between two groups.

3. Results

3.1. Overexpression of CHIP down-regulates the protein level of iNOS in RAW264.7 cells

Previous studies showed that CHIP incorporated into the eNOS/Hsp90 complex and specifically decreased soluble eNOS levels in COS cells [20], and CHIP acted as an E3 ligase for nNOS to enhance the ubiquitination and proteasomal degradation of nNOS in HEK293T cells [15]. However, little is known about the mechanism utilized by CHIP to regulate iNOS protein level. Because iNOS expression is stimulated by some inflammatory stimuli, we first observed whether CHIP could decrease high level of iNOS protein induced by lipopolysaccharide (LPS) in RAW264.7 cells. RAW264.7 cells were transiently transfected with Myc-tagged CHIP followed by stimulated with LPS (100 ng/ml) and endogenous iNOS level was measured by Western blot analysis. As same as previous report [20], iNOS protein up-regulated by LPS significantly declined in CHIP over-expressed cells compared with those cells transfected with empty expression vectors (Fig. 1).

![Fig. 1](image)
3.2. Effects of CHIP on iNOS protein level in HEK293 cells

HEK293 cell line is an epithelial cell line that does not express any of the NOS genes and has been used extensively to study exogenously expressed iNOS [6]. To further obtain effect of CHIP on iNOS turnover, we co-transfected pcDNA3-iNOS and Myc-CHIP plasmids into HEK293 cells and examined the level of iNOS with Western blotting. The results showed that coexpression of CHIP led to a considerable reduction in the level of iNOS compared to pcDNA3 control in HEK293 cells (Fig. 2A).

To further confirm the effects of CHIP on iNOS protein level, CHIP-specific small interference RNA (siRNA) was transfected into HEK293 cells followed by iNOS transfection. The successful knockdown of the CHIP gene was exhibited by the specific reduction of the CHIP expression in pBS-U6-CHIP but not pBS-U6 transfected HEK293 cells (Fig. 2B). As expected, iNOS level in CHIP knockdown cells was apparently higher than control cells (Fig. 2B). Furthermore, transfecting CHIP siRNA to inhibit CHIP overexpression significantly reversed CHIP-induced iNOS level reduction (Fig. 2C). These data indicated that CHIP down-regulated iNOS in HEK293 cells.

3.3. CHIP attenuates the stability of iNOS

In order to analyze how CHIP down-regulated iNOS, we next determined the effects of CHIP on stability of iNOS. pcDNA3-iNOS and Myc-CHIP were co-transfected into HEK293 cells and 36 h after transfection cells were treated with 2.0 μg/ml cycloheximide (CHX), a broad-spectrum protein synthesis inhibitor, for indicated period. The cell lysates were then subjected to Western blot analysis with anti-iNOS and anti-Myc antibodies. The results showed that iNOS protein level decreased significantly in CHIP over-expressed cells compared with control cells, indicating that CHIP attenuated the stability of iNOS by reducing the half-life of iNOS (Fig. 3).

3.4. CHIP promotes the proteasomal degradation and enhances the ubiquitination of iNOS

Above results showed that CHIP shortened iNOS half-life suggesting that CHIP could promote iNOS degradation. We then focused on the effects of CHIP on proteasomal degradation of iNOS. HEK293 cells were co-transfected with pcDNA3-iNOS and Myc-CHIP and 36 h after transfection cells were treated with the proteasome inhibitor MG132 (30 μM) for additional 8 h. Cell lysates were subjected to Western blot with anti-Myc and anti-iNOS antibody, respectively. As shown in Fig. 4A, CHIP-induced iNOS decrease was partially reversed by MG132 treatment, which suggested that the effects of CHIP on iNOS reduction were proteasome dependent. To confirm this result, expression plasmids of Myc-CHIP, HA-ubiquitin and pcDNA3-iNOS in various combinations were co-transfected into HEK293 cells. Data showed that expression of ubiquitin

Fig. 2. Effects of CHIP on iNOS protein level in HEK293 cells. (A) HEK293 cells were transfected with pcDNA3-iNOS (0.8 μg) and various concentrations of Myc-CHIP plasmids (0, 0.2, 0.5 and 0.8 μg). And after 36 h, cell lysates were subjected to Western blot with anti-iNOS, anti-Myc and anti-GAPDH antibodies. (B) HEK293 cells were transfected with pBS-U6-CHIP constructs or pBS-U6 as control and after 36 h, cells were transfected with iNOS. Thirty-six hours after second transfection, the Western blot analysis was performed by using anti-iNOS, anti-CHIP or anti-GAPDH antibody as indicated. (C) HEK293 cells were transfected with Flag-GFP constructs together with pBS-U6-CHIP or pBS-U6. After 36 h, cells were transfected with iNOS and Myc-CHIP for additional 36 h. Western blot was performed using antibodies to iNOS, Myc, Flag and GFP (transfection control). Each bar represents the means ± SD of three experiments. *p < 0.05; **p < 0.01.
enhanced CHIP-induced iNOS decrease indicating further that CHIP down-regulated iNOS by promoting proteasome degradation (Fig. 4B).

As ubiquitination of iNOS is considered to be necessary for its degradation [9], we next observed if CHIP could affect the ubiquitination of iNOS. pcDNA3-iNOS together with or without Myc-CHIP was transfected into HEK293 cells, and 36 h after transfection cells were subjected to immunoprecipitated with iNOS antibody followed by Western blot analysis using antibody to iNOS or ubiquitin, respectively. Results showed that overexpression of CHIP promoted iNOS ubiquitination (Fig. 5A). We then co-transfected the indicated combinations of expressing vectors encoding iNOS, Myc-CHIP and HA-ubiquitin into HEK293 cells and performed Western blotting of iNOS immunopellets using anti-HA antibody. The result showed ubiquitin increased iNOS ubiquitination was significantly enhanced by CHIP (Fig. 5B). All these data indicated that CHIP promoted iNOS ubiquitination and proteasomal degradation.

3.5. CHIP physically associates with iNOS

Previous studies have shown that iNOS can interact with HSP70 or HSP90 [26,27], and CHIP interacts with these molecular chaperones and their clients to result in the client substrates ubiquitylation and degradation [15,17,19]. We speculated that CHIP could modulate iNOS ubiquitination and degradation by interacting with iNOS. RAW264.7 cells were treated with LPS (100 ng/ml) for 12 h and cell lysates were immunoprecipitated by CHIP antibody or rabbit IgG antibody as control. The Western blot analysis of precipitates with anti-iNOS or anti-CHIP antibody demonstrated that iNOS combined with CHIP in RAW264.7 cells (Fig. 6A). We further detected the protein binding between CHIP and iNOS in overexpression conditions. HEK293 cells were transiently transfected with HA-CHIP and Myc-iNOS and 36 h after transfection cells were subjected to immunoprecipitation using anti-HA antibody followed by Western blot with anti-Myc or anti-HA antibody. Whole cell lysates were analyzed by Western blotting with anti-HA or anti-Myc monoclonal antibody. The results showed that exogenous CHIP also associated with iNOS, although iNOS has been down-regulated by over-expressed CHIP (Fig. 6B). Therefore, the data from the observation of either endogenous or over-expressed CHIP and iNOS indicated that CHIP physically associated with iNOS in cells.

3.6. CHIP decreases iNOS induced release of NO

NO is an important inflammation product generated by iNOS and primarily involves in promoting inflammatory response [28].
Because CHIP promoted ubiquitination and degradation of iNOS, we thus examined the effect of CHIP on LPS release from RAW264.7 cells by detecting changes in nitrite concentration in cell culture media. RAW264.7 cells were transfected with Myc-CHIP and then were treated with 100 ng/ml LPS. As shown in Fig. 7A, NO level in CHIP transfected cells was lower than those cells transfected with control vectors. Furthermore, we co-transfected Myc-CHIP and iNOS into HEK293 cells and analyzed the NO level by nitrite analysis. As expected, overexpression of CHIP also reduced the production of NO induced by iNOS (Fig. 7B).

4. Discussion

iNOS has been implicated in the pathogenesis of many diseases, including Alzheimer’s disease, tuberculosis, asthma, transplant rejection, stroke, glaucoma, inflammatory bowel disease, arthritis, and septic shock [29–31]. Such wide implication has produced a corresponding intense interest in understanding the regulation of NO synthesis by iNOS, with the goal of developing therapeutic strategies aimed at selective modulation of iNOS activity. Synthesis, catalytic activity, and degradation are three regulatory targets for controlling the activity of the enzyme. Since the protein degradation is thought to affect the level of its gene expression in cells, thus, understanding the mechanisms and regulation of iNOS degradation are important for revealing how cells control the level of NO synthesis during inflammation and host defense [9].

Ubiquitination and proteasomal degradation is a critical component of protein quality control in cells. The E3 ubiquitin ligases are widely thought to select target proteins for ubiquitination and thus commit the target protein to proteasomal degradation. CHIP is a U-box-containing E3 ubiquitin ligase that binds through its TPR domain to independent TPR acceptor sites on HSP90 and HSP70 and mediates ubiquitination of other HSP90 chaperoned proteins [12,13].

Among three NOS isoforms, nNOS is reported to be ubiquitinated and degraded by CHIP [15], but eNOS, another isoform of NOS, is regulated by CHIP in intracellular trafficking through partitioning.
ing the soluble enzyme into the insoluble (inactive) cellular compartment rather than in eNOS degradation [20]. This difference of the two enzymes in trafficking versus degradation may relate to the differences in the structure of the N-termini, which include a PDZ domain in nNOS but not eNOS and myristylation and palmitylation sites on eNOS but not nNOS. However, neither PDZ domain nor myristylation and palmitylation sites are appeared in the N-terminal of iNOS. Furthermore, similar with nNOS, iNOS also associates with HSP90 and HSP70 [26,27]. We reasonably suppose that iNOS can be subjected to ubiquitination and proteasomal degradation by CHIP. In fact, it has been shown that transforming growth factor-β (TGF-β) could enhance iNOS degradation [32] and deoxymethasone inhibited iNOS activity partially by enhancing iNOS degradation [33]. Besides, Felley-Bosco et al. reported that in human colon carcinoma cells, caveolin-1 down-regulates iNOS via the proteasome pathway [34]. As expected, in the current study, the data both from CHIP overexpression and RNAi experiments demonstrate that CHIP reduces cellular iNOS protein level lifted by LPS stimulation or overexpression of iNOS gene. Mechanically CHIP interacts with iNOS to promote ubiquitination and degradation of iNOS, and consequently reduces the iNOS induced NO production. These results reveal a novel role of CHIP in inhibiting iNOS induced over-production of NO, and provide a new insight for understanding the mechanism of iNOS degradation and regulation of iNOS activity.

Up till now, iNOS inhibitors have attained widespread use as anti-inflammatory agents, such as L-NNA, L-NMMA and 1400W [35,36]. The regulation of iNOS activity is also a target for anti-inflammatory therapy. Our results strongly suggest that CHIP may act as a chaperone-dependent ubiquitin ligase for iNOS and these findings provide a new insight for realizing the molecular mechanism by which iNOS degradation is regulated.

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