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Inducible nitric oxide synthase aggresome formation is mediated by nitric oxide

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ABSTRACT

Nitric oxide (NO) generated by inducible NO synthase (iNOS) contributes critically to inflammatory injury and host defense. While previously thought as a soluble protein, iNOS was recently reported to form aggresomes inside cells. But what causes iNOS aggresome formation is unknown. Here we provide evidence demonstrating that iNOS aggresome formation is mediated by its own product NO. Exposure to inflammatory stimuli (lipopolysaccharide and interferon-γ) induced robust iNOS expression in mouse macrophages. While initially existing as a soluble protein, iNOS progressively formed protein aggregates as a function of time. Aggregated iNOS was inactive. Treating the cells with the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) blocked NO production from iNOS without affecting iNOS expression. However, iNOS aggregation in cells was prevented by L-NAME. The preventing effect of NO blockade on iNOS aggresome formation was directly observed in GFP-iNOS-transfected cells by fluorescence imaging. Moreover, iNOS aggresome formation could be recaptured by adding exogenous NO to L-NAME-treated cells. These studies demonstrate that iNOS aggresome formation is caused by NO. The finding that NO induces iNOS aggregation and inactivation suggests aggresome formation as a feedback inhibition mechanism in iNOS regulation.

1. Introduction

Nitric oxide (NO) is dubbed as a double-edged sword for it can cause opposite biological effects depending on its concentrations [1–4]. Physiological levels of NO function as a signaling molecule in regulating neuronal and cardiovascular activities. However, being a free radical, excessive amounts of NO can damage cells. Thus, NO has been implicated in inflammatory injury in various diseases. On the other hand, the destructive feature of NO is also utilized in host defense against microbe invaders. In mammals, NO is produced by a family of NO synthase (NOS) including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [5–7]. Among them, iNOS is primarily responsible for NO production in cell injury and host defense [8,9]. All NOS isoforms use L-arginine, oxygen, and NADPH as co-substrates to synthesize NO. Compared to the other two isoforms, iNOS possesses the highest NO-generating potency and this feature is thought to be ideal for its function in host defense.

nNOS and eNOS constitutively exist in cells, whereas little iNOS can be detected in normal cells and tissues. iNOS expression is induced by inflammatory mediators. Bacterial product lipopolysaccharide (LPS) and cytokines, such as interferon-γ (IFN-γ), are potent inducers of iNOS expression. Once expressed, iNOS has high binding affinity with its catalytic cofactor calmodulin and exhibits constant activity [10,11]. Thus, NO production from iNOS is largely determined by the levels of iNOS proteins. It has been thought that iNOS stays as a soluble and stable protein in the cytosol. However, recent studies report that iNOS forms aggresomes inside cells [12,13]. Aggresome formation deactivates iNOS and this is proposed as a possible mechanism to down-regulate NO production in inflammation. But an important question remains regarding what causes iNOS aggresome formation. In this present study, we provide evidence demonstrating that NO per se causes iNOS aggresome formation. Blocking NO production prevents iNOS aggresome formation. This finding may shed new light on the long-sought mechanism of the feedback inhibition of iNOS by NO.

2. Materials and methods

2.1. Materials

Cell culture materials were purchased from Invitrogen (Carlsbad, CA). Anti-iNOS antibody was obtained from BD Transduction

Abbreviations: NO, nitric oxide; iNOS, inducible NO synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ; L-NAME, *N*-nitro-L-arginine methyl ester; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine.

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Laboratories (Franklin Lakes, NJ). LPS, recombinant mouse IFN-γ, *N*-nitro-L-arginine methyl ester (L-NAME) and anti-GAPDH antibody were products of Sigma (St. Louis, MO). *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) was purchased from Biomol (Plymouth Meeting, PA). Unless otherwise indicated, all other chemicals used in this study were purchased from Sigma.

2.2. Cell culture

Mouse macrophages (RAW264.7, ATCC) and human embryonic kidney 293 (HEK293) 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 RAW264.7 cells was induced by LPS (2 μg/ml, serotype 026:B6) and IFN-γ (100 U/ml) [14].

2.3. Western blot analysis

Cells were harvested and lysed in lysis buffer (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 30 min incubation on ice, lysates were centrifuged at 14,000*g* for 15 min at 4 °C. The supernatants and pellets were recovered as soluble and insoluble fractions, respectively. Protein concentrations of soluble fractions were determined by using the detergent-compatible protein assay kit (Bio-Rad). The insoluble pellets were washed by PBS. After 5 min boiling in 1 × 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).

2.4. iNOS activity assay

iNOS activity was measured by the L-[^{14}C]arginine to L-[^{14}C]citrulline conversion assay. iNOS expression was induced in RAW 264.7 by LPS/IFN-γ. After 12-h induction, cells were homogenized in homogenate buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture). The homogenate was then centrifuged (14,000*g* for 30 min at 4 °C), and the supernatants were recovered and used for measuring soluble iNOS activity. To obtain aggregated iNOS generated by NO accumulation, cells were incubated with iNOS inducers (LPS/IFN-γ) for 30 h, and then 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 calmodulin, 10 μM BH_4, 0.1 μM L-[^{14}C]arginine, and 18 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.

2.5. Nitrite assay

Total nitrite released in cell culture medium was measured with a Griess reagent kit (Invitrogen). The reaction consisted of 20 μl of Griess Reagent, 150 μl of medium, and 130 μl of deionized water. After incubation of the mixture for 30 min at room temperature, nitrite levels were measured at 548 nm using a M2 spectrophotometric microplate reader (Molecular Devices).

2.6. Plasmid and transient transfection

The cDNA encoding murine iNOS was subcloned into the pEGFP-C3 vector and then transfected into HEK293 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, pEGFP-C3/iNOS plasmid and lipofectamine were mixed in Opti-MEM media (Invitrogen) and added to 50% confluent cells. After 4-h incubation, serum was added back to allow cell recovery.

2.7. Fluorescence imaging

HEK293 cells were transfected with pEGFP-C3/iNOS plasmid in the presence and absence of L-NAME (2 mM) for 26 h at 37 °C. Images were then acquired with a Zeiss Axioskop 40 Microscope equipped with a Nikon DS-Qi1 Monochrome Digital Camera.

2.8. Statistical analysis

Data are expressed as mean ± SE. Comparisons are made using a two-tailed Student's unpaired *t* test. Differences are considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. iNOS aggregation in mouse macrophages

To determine the mechanism of iNOS aggresome formation, we first characterized iNOS aggregation process in mouse macrophages (RAW264.7) exposed to LPS/IFN-γ. After LPS/IFN-γ induction, cells were fractioned into supernatant (soluble) and pellet (insoluble) fractions. We monitored the levels of iNOS in both soluble and pellet fractions. As shown in Fig. 1A, at the early phase of induction, iNOS was only seen in the soluble fractions of cells. However, after 22 h of LPS/IFN-γ induction, soluble iNOS was gradually lost. Corresponding to the loss of iNOS in the soluble fractions, progressive iNOS accumulation was seen in the pellet fractions (Fig. 1A and B). After 28-h induction, iNOS was mostly seen in the pellet fractions. These observations were consistent with those reported in the prior literature and indicated that iNOS formed aggresomes. Aggregation often causes proteins to lose their functions. To determine the effect of aggregation on iNOS function, we compared the activity of soluble and aggregated iNOS with the L-[^{14}C]arginine to L-[^{14}C]citrulline conversation assay. As expected, aggregated iNOS exhibited little catalytic activity (Fig. 1C). These data demonstrated that iNOS formed aggregates as a function of time in macrophages and this led to enzyme deactivation.

3.2. Inhibition of NO production prevented iNOS aggregation

iNOS is a high-output NO-generating enzyme. We hypothesized that NO might play a role in iNOS aggresome formation. To examine such a hypothesis, iNOS expression was induced in the absence and presence of L-NAME, an L-arginine derivative that selectively blocks NOS function [5]. As shown in Fig. 2A, L-NAME treatment had no effect on iNOS expression in LPS/IFN-γ-stimulated cells. Remarkably, blocking NO production with L-NAME prevented iNOS aggregation in cells. The relationship between NO production and iNOS aggregation was clearly demonstrated in Fig. 2B. These data suggested that NO played essential role in iNOS aggresome formation.

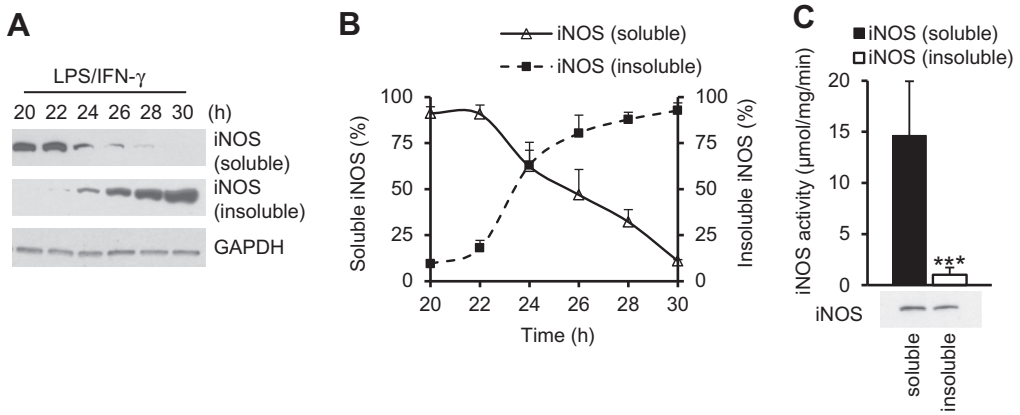

Fig. 1. iNOS aggregation in mouse macrophages after LPS/IFN-γ stimulation. (A) iNOS expression was induced in RAW264.7 cells by LPS (2 μg/ml)/IFN-γ (100 U/ml). As shown, progressive iNOS aggregation was seen in cells after 22-h induction. (B) Quantitative analyses of iNOS distribution in the soluble and insoluble fractions. Data are means ± SE, $n = 4$. (C) The catalytic activity of iNOS was lost after aggregation. $^{***}P < 0.01$, $n = 4$.

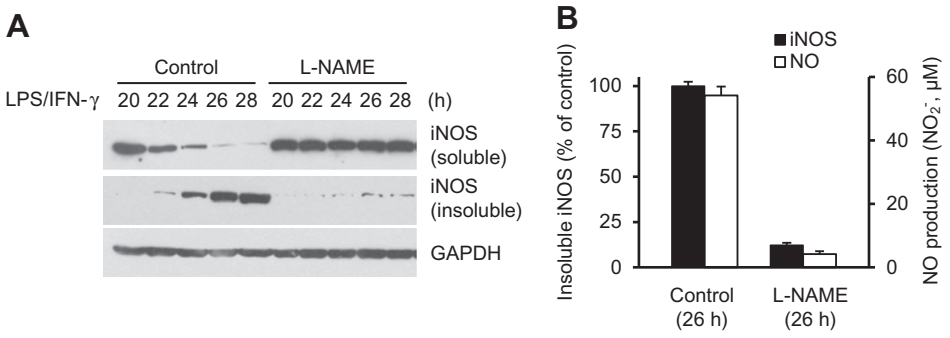

Fig. 2. Blocking NO production prevented iNOS from aggregation. (A) iNOS aggregation was prevented by blocking iNOS function with L-NAME (2 mM). (B) Quantitative analyses of the effects of L-NAME on iNOS aggregation and NO production in cells after LPS/IFN-γ induction for 26 h. Data are means ± SE, $n = 4$.

3.3. Demonstration of NO-mediated GFP-iNOS aggresome formation in cells

To visualize the role of NO in iNOS aggresome formation, we constructed a GFP-tagged iNOS expression vector and transfected it into HEK293 cells. Consistent with the cell fractionation studies, GFP-iNOS formed aggresomes in cells after 26-h transfection (Fig. 3, left panel). In the presence of L-NAME, iNOS was evenly distributed in the cytosol and no aggresome formation was detected (Fig. 3, right panel). This data strongly indicated that NO caused iNOS aggresome formation.

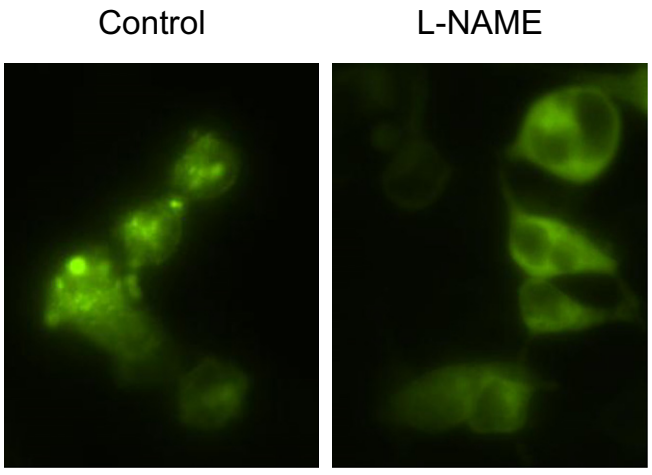

Fig. 3. Fluorescence imaging of GFP-iNOS aggresome formation in the absence and presence of NOS inhibitor L-NAME. GFP-iNOS fusion proteins were expressed in HEK293 cells. As shown, GFP-iNOS formed aggresomes in control cells in the absence of L-NAME. In the presence of L-NAME, a uniform distribution of GFP-iNOS was seen in the cytosol. Representative images are shown from triplicate experiments.

3.4. Exogenous NO induced iNOS aggregation in L-NAME-treated cells

Finally, we examined whether iNOS aggregation could be recaptured by exposing L-NAME-treated cells to exogenous NO. As shown in Fig. 4A, iNOS stayed as a soluble protein in L-NAME-treated cells. However, exposing the L-NAME-treated cells to NO donor SNAP [15–17] induced iNOS aggregation in a time-dependent manner. The effects of exogenous NO on iNOS aggregation in L-NAME-treated cells were summarized in Fig. 4B and C. These data further confirmed the crucial role of NO in iNOS aggresome formation.

Among the NOS isoforms, nNOS and eNOS have been shown to be anchored to the plasma membrane through the interactions with various adaptor proteins [5,7]. iNOS, however, has been thought to exist as a soluble protein in the cytosol until recent reports from the Eissa group, in which they show that iNOS forms aggresomes [12,13]. They transfected GFP-iNOS into HEK293 cells and found that iNOS formed aggresomes. iNOS aggresome formation was also reported in cytokine-stimulated bronchial epithelial cells by the same group as well as mouse macrophages in the present study. However, the key question is what causes iNOS aggresome formation. The present study shows, for the first time, that it is the NO per se that causes iNOS aggregation. Blocking NO pro-

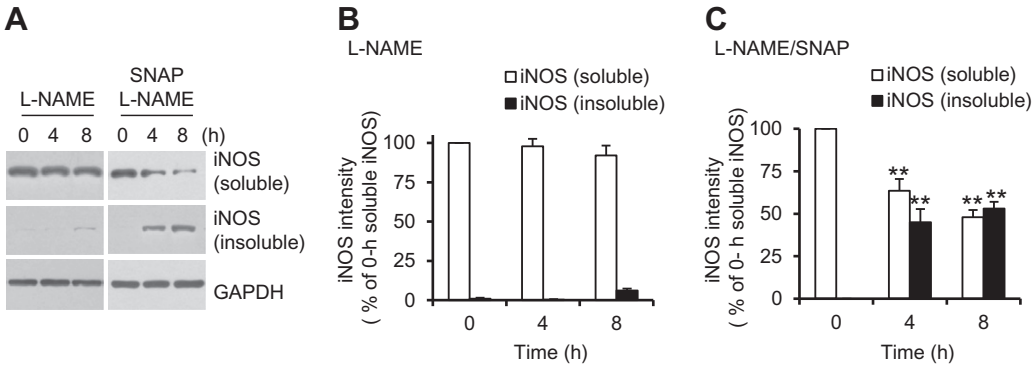

Fig. 4. Exogenous NO induced iNOS aggregation in L-NAME-treated cells. (A) Under iNOS inhibition (L-NAME, 2 mM), iNOS stayed as a soluble protein inside cells. Exposing these L-NAME-treated cells to exogenous NO (generated by NO donor SNAP, 2 mM) triggered iNOS aggregation. (B and C) Quantitative analyses of soluble and insoluble iNOS in L-NAME-treated cells in the absence and presence of NO donor. Data are means ± SE, $n = 3–5$.

duction has no effect on iNOS expression but prevents iNOS aggresome formation in both native and transfected cells. Soluble iNOS is a constantly active enzyme. iNOS in aggresomes, on the other hand, is inactive. It is known that NO can cause feedback inhibition to iNOS, but the mechanism has been elusive. The current discovery that NO induces iNOS aggregation and subsequent deactivation may be the long-sought mechanism for the feedback inhibition of iNOS by NO.

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