Inducible Nitric-oxide Synthase Is Regulated by the Proteasome Degradation Pathway*

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Inducible nitric-oxide synthase (iNOS) is responsible for nitric oxide (NO) synthesis from L-arginine in response to inflammatory mediators. To determine the degradation pathway of iNOS, human epithelial kidney HEK293 cells with stable expression of human iNOS were incubated in the presence of various degradation pathway inhibitors. Treatment with the proteasomal inhibitors lactacystin, MG132, and N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal resulted in the accumulation of iNOS, indicating that these inhibitors blocked its degradation. Moreover, proteasomal inhibition blocked iNOS degradation in a dose- and time-dependent manner as well as when NO synthesis was inhibited by N^3-nitro-L-arginine methyl ester. Furthermore, proteasomal inhibition blocked the degradation of an iNOS splice variant that lacked the capacity to dimerize and of an iNOS mutant that lacks L-arginine binding ability, suggesting that iNOS is targeted by proteasomes, notwithstanding its capacity to produce NO, dimerize, or bind the substrate. In contrast to proteasomal inhibitors, the calpain inhibitor calpastatin and the lysosomal inhibitors trans-eoxysuccinyl-L-leucylamido-4-guanidino butane, leupeptin, pepstatin-A, chloroquine, and NH_4Cl did not lead to significant accumulation of iNOS. Interestingly, when cytokines were used to induce iNOS in RT4 human epithelial cells, the effect of proteasomal inhibition was dichotomous. Lactacystin added prior to cytokine stimulation prevented iNOS induction by blocking the degradation of the NF-kB inhibitor IкB-α, thus preventing activation of NF-kB. In contrast, lactacystin added 48 h after iNOS induction led to the accumulation of iNOS. Similarly, in murine macrophage cell line RAW 264.7, lactacystin blocked iNOS degradation when added 48 h after iNOS induction by lipopolysaccharide. These data identify the proteasome as the primary degradation pathway for iNOS.

Nitric oxide (NO), an important signaling and cytotoxic molecule, is synthesized from L-arginine by isoforms of nitric-oxide synthase (NOS) (1, 2). As a signaling molecule, NO is produced by two constitutive Ca^{2+}-dependent isoforms, neuronal and endothelial NOS (or NOS1 and NOSIII, respectively). Ca^{2+}-activated calmodulin binds to and transiently activates constitutive NOS dimers (2, 3). Because of the transient nature of elevated Ca^{2+} levels, the activity of NO produced is short-lived. As an agent of inflammation and cell-mediated immunity, NO is produced by a Ca^{2+}-independent cytokine-inducible NOS (iNOS or NOSII) that is widely expressed in diverse cell types under transcriptional regulation by inflammatory mediators (4–6). Calmodulin is tightly bound to iNOS even at basal Ca^{2+} levels, and therefore iNOS is notably distinguished from the constitutive isoforms by its prolonged production of a relatively large amount of NO (7). iNOS has been implicated in the pathogenesis of many diseases including Alzheimer’s disease, pulmonary tuberculosis, asthma, lung cancer, transplant rejection, cerebral infarct, glaucoma, bacterial pneumonia, inflammatory bowel disease, arthritis, and septic shock (8–10).

The activity of an enzyme can be controlled through the regulation of its synthesis, catalytic activity, or degradation. Although much is known about factors affecting the synthesis and catalytic activity of iNOS, little is known about its degradation. The 26S proteasome is a large multisubunit protease responsible for the selective degradation of a number of short-lived regulatory proteins, the activity of which must be tightly regulated, such as NF-κB, STAT1, fos/jun, and cyclins (11–13). In this report, we identify the 26S proteasome as the major pathway responsible for human iNOS degradation. Human iNOS is targeted for degradation by the proteasome, notwithstanding its capacity to produce NO, dimerize, or bind the substrate. Furthermore, we demonstrate that the proteasome pathway regulates human iNOS at both transcriptional and post-translational levels.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Lactacystin, calpastatin, and taurocholic acid were obtained from Calbiochem-Novabiochem. N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN), N-carbobenoxozyl-L-leucinyl-L-leucinyl-L-norleucinal (MG132), trans-eoxysuccinyl-L-leucylamido-4-guanidino butane, leupeptin, pepstatin-A, chloroquine, NH_4Cl, N-ω-tosyl-ω-lysine chloromethyl ketone, N-ω-tosyl-L-phenylalanine chloromethyl ketone, and lipopolysaccharide (LPS) were from Sigma. All other reagents were purchased from either Fisher or sources stated in the text.

**Antibodies**—IE8-B8 (Research and Diagnostic Antibodies) is a monoclonal antibody specific for the iNOS isoform and can detect both human and murine iNOS (8, 14). The monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase was from Advanced Immunochemical. A goat polyclonal antibody (Santa Cruz Biotechnology) and a rabbit polyclonal antibody (New England Biolabs) were used in immunoblotting for the detection of IкB-α and phosphorylated IкB-α, respectively.

**Cell Culture**—Human embryonic kidney (HEK) 293, human bladder iNOS, t-NAME, N^3-nitro-L-arginine methyl ester; iNOS-8, -9, iNOS splice variant with deletion of exons 8 and 9.

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transitional cell papilloma (RT4), and murine macrophage RAW 264.7 cell lines were purchased from the American Type Culture Collection. The cells were cultured in phenol red-free improved minimal essential medium (HEK293), Dulbecco’s modified Eagle’s (RAW 264.7), or McCoy’s media (RT4) supplemented with 2 mM glutamine and 10% heat-inactivated filtered (40 nm-filter) fetal bovine serum (HyClone Laboratories, Inc.) at 37 °C in 5% CO₂.

Transfections—Human iNOS cDNA, inserted into the expression vector pRC/CMV (Invitrogen) under the control of the cytomegalovirus promoter, was used. Cationic lipid-mediated transient transfection was done using LipofectAMINE and a transfection-enhancing Plus reagent. Cationic lipid-mediated transient transfection was done using LipofectAMINE and a transfection-enhancing Plus reagent. In parallel experiments, cells were incubated in the presence of vehicles used to dissolve the corresponding inhibitors. A, cells were harvested by lysis on ice for 45 min in 40 mM bis-Tris propane buffer, pH 7.7, 150 mM NaCl, and 10% glycerol with 20 mM sodium taurocholate. In some experiments, to lyse cells and precipitate proteins immediately, 20% trichloroacetic acid (B) or 1% boiling SDS (C) was added directly to the cells. Cell lysates (50 μg) were subjected to SDS/polyacrylamide gel electrophoresis followed by immunoblotting with an anti-human iNOS antibody. The results are representative of three independent experiments performed in duplicate.

RESULTS AND DISCUSSION

Human iNOS Is a Target for the Proteasome Degradation Pathway—We investigated the degradation pathway for iNOS in the HEK293 cell line, an epithelial cell line that does not express any of the NO genes and has been used extensively to study exogenously expressed human iNOS (14, 17). We had previously produced an HEK293 cell line with stable expression of human iNOS (14), henceforth referred to as HEK293-iNOS. We tested the effects of the potent yet nonspecific proteasome inhibitors, ALLN and MG132, and the highly specific proteasome inhibitor lactacystin on iNOS expression in this cell line (13, 18). Cells were incubated with proteasome inhibitors or the corresponding vehicle for 20 h and then harvested by lysis on ice (see “Experimental Procedures”) (14). Lactacystin, ALLN, and MG132 blocked human iNOS degradation leading to its accumulation, indicating that the effect of proteasomal inhibition on iNOS was not the result of an in vitro effect during cell lysis.

The Specific Proteasome Inhibitor Lactacystin Blocks Human iNOS Degradation in a Dose- and Time-dependent Manner—To further characterize human iNOS degradation via the proteasome pathway, we examined the dose and time dependence of the effect of lactacystin on iNOS expression. Incubation of concentrations were determined by using a bicinchoninic acid reagent (Pierce). Cell lysates (50 μg) were mixed with one-third volume of 4× Laemmli sample buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 0.004% bromphenol blue, 40% glycerol, and 400 mM dithiothreitol), boiled at 95 °C for 5 min, and then subjected to SDS/polyacrylamide gel electrophoresis. After SDS/polyacrylamide gel electrophoresis, immunoblotting was done with the 1E8-B8 antibody. An enhanced chemiluminescence system was used for detection (SuperSignal West Pico, Pierce). Images were acquired using a cooled charge-coupled device camera (Eagle Eye II Still Video System, Stratagene).

iNOS Degradation by the Proteasome Pathway—To further characterize human iNOS degradation via the proteasome pathway, we examined the dose and time dependence of the effect of lactacystin on iNOS expression. Incubation of...
HEK293-iNOS cells for 20 h with increasing concentrations of lactacystin (1–20 μM) resulted in a corresponding increase in iNOS accumulation (Fig. 2). In similar experiments, incubation of HEK293-iNOS cells with as little as 2 μM lactacystin for variable times (8–32 h) resulted in a time-dependent accumulation of iNOS in HEK293-iNOS cells (Fig. 3). Increase in iNOS expression could be detected as early as 8 h after lactacystin addition and continued to accumulate further over the time course of the study.

**Lactacystin Blocks Human iNOS Degradation Independent of NO Production by iNOS**—In addition to its role in the degradation of regulatory proteins, the 26S proteasome has been implicated in the removal of faulty, misfolded, and oxidatively inactivated cellular proteins (12). In this context, we investigated whether the proteasomal degradation of human iNOS depends on NO production by iNOS. We tested the effect of lactacystin on iNOS expressed in HEK293-iNOS cells incubated in the presence or absence of the substrate analogue NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME) (19). Incubation of the cells in the presence of 3 mM L-NAME resulted in the inhibition of NO synthesis by iNOS as estimated by measuring nitrite accumulation in culture media (data not shown). Incubation of the cells with 10 μM lactacystin for 20 h blocked iNOS degradation in the presence or absence of L-NAME (Fig. 4). Similar results were obtained when the specific iNOS inhibitor, S-ethylisothiourea (50 μM), was used instead of L-NAME (data not shown) (20). These data suggest that the ability of iNOS to synthesize NO is not required for its targeting by the proteasome degradation pathway.

**Lactacystin Blocks Degradation of a Splice Variant and a Mutant of Human iNOS That Lack the Capacity for Dimerization or Substrate Binding**—Like all NOS isoforms, the synthesis of NO by human iNOS is active only in its dimer form (2, 17). We previously identified a splice variant of human iNOS that lacks exons 8 and 9 (iNOS<sup>Δ8,9</sup>), a domain critical for dimerization and NO synthesis (14). Therefore,
iNOS\textsuperscript{8,9} and iNOS\textsubscript{D290A} (Fig. 5). These results suggest that the targeting of human iNOS by the proteasome degradation pathway is independent of the capacity of iNOS to dimerize or bind the substrate.

Inhibitors of Calpains, Trypsin-like Proteases, and Chymotrypsin-like Proteases and Lysosomal Inhibitors Do Not Block Human iNOS Degradation—To investigate the roles of other cellular proteolytic pathways that might be involved in iNOS degradation, the effect of various degradation inhibitors was tested. After a 24-h incubation in the presence of the specific inhibitor, the cells were immediately lysed by boiling in SDS-containing buffer. In contrast to proteasomal inhibitors, the calpain inhibitor (calpastatin, 5 \textmu M), the trypsin-like protease inhibitor (N-\textalpha-p-tosyl-l-lysine chloromethyl ketone, 100 \textmu M), or the chymotrypsin-like protease inhibitor (N-\textalpha-p-tosyl-l-phenylalanine chloromethyl ketone, 50 \textmu M) did not lead to the accumulation of iNOS (Fig. 6A). Similar results were obtained using the lysosomal protease inhibitors trans-epoxysuccinyl-l-leucylamido-4-guanidino butane (50 \textmu M) and leupeptin (50 \textmu M), the aspartate protease inhibitor pepstatin-A (100 \textmu M), and the lysosomal acidification inhibitors chloroquine (100 \textmu M) or NH\textsubscript{4}Cl (20 mM) (Fig. 6, B and C). Yet, there was a mild but not consistent increase in iNOS detection with lysosomal inhibitors (Fig. 6). Because of the mild increase seen in some of the iNOS immunoblot signals in Fig. 6, a possible role for the lysosomal pathway in human iNOS degradation cannot be ruled out. As a positive control, lactacystin was used during the same experiments and resulted in a more pronounced accumulation of iNOS, suggesting that the proteasome pathway plays a primary role in human iNOS degradation.

The Proteasome Regulates iNOS Expression at Transcriptional and Post-translational Levels; Lactacytin Prevents iNOS Induction by Cytokines—NF-\kappaB is a major transcription factor involved in the inducible expression of iNOS (22, 23). It is a ubiquitous transcription factor that is activated by a myriad of proinflammatory stimuli and cytokines (24). Proinflammatory stimuli activate NF-\kappaB through a tightly regulated cascade of phosphorylation, ubiquitination, and proteasomal proteolysis of a physically associated class of inhibitor molecules, the best characterized of which is I\kappaB-\alpha. Therefore, proteasomal inhibitors block the degradation of already phosphorylated and ubiquitinated I\kappaB-\alpha, thus aborting NF-\kappaB activation (25). Inhibitors of the proteasome pathway were found to interfere with the induction of iNOS in rat alveolar macrophages by blocking NF-\kappaB activation (26). We hypothesized that the proteasome regulates iNOS on two distinct levels: at the transcriptional level, by degrading I\kappaB-\alpha and thus activating NF-\kappaB, and at the post-translational level by degrading iNOS protein. To test this hypothesis we used a cytokine mixture of IL-1\beta (0.5 ng/ml), tumor necrosis factor-\alpha (10 ng/ml), and IL-6 (200 units/ml) to induce iNOS expression in RT4 cells (6, 15). The addition of 40 \textmu M lactacystin 1 h prior to stimulation prevented iNOS induction (Fig. 7A). The inhibitory effect on iNOS induction was caused by the inhibition of I\kappaB-\alpha degradation, as suggested by increased levels of I\kappaB-\alpha (Fig. 7B). The increase in I\kappaB-\alpha seen with proteasomal inhibition was caused by inhibition of the degradation of already phosphorylated iNOS.
ated IkB-α, as demonstrated by immunoblotting cell lysates with an anti-phospho-IkBα-specific antibody (Fig. 7C) (27).

Proteasomal Inhibition by Lactacystin Blocks iNOS Degradation in RT4 Cells—We hypothesized that in cells stimulated by cytokines to produce iNOS, once iNOS is produced, proteasomal inhibitors will block its degradation. To test this hypothesis, RT4 cells were incubated for 24 h in the presence of the same cytokine mixture used above followed by a 24-h incubation with fresh medium without cytokines and then in the presence of lactacystin (20–40 μM for 20 h). The treatment of cells with lactacystin led to a dose-dependent accumulation of iNOS (Fig. 8), similar to that seen in experiments with transfected HEK293 cells expressing iNOS.

Thus, there is a dichotomous effect of proteasomal inhibition on cytokine-induced human iNOS. This phenomenon may have contributed to the lack of prior reports on human iNOS degradation. The finding that cytokine-induced iNOS in RT4 cells is degraded through the proteasome pathway indicates that the role of the proteasome pathway in iNOS degradation is not peculiar to HEK293 cells, nor is it likely to be simply a consequence of overexpression.

Proteasomal Inhibition Blocks Murine iNOS Degradation in RAW 264.7 Cells—To extend the above observations to murine cells, we tested the effect of proteasomal inhibition on murine iNOS degradation. The murine macrophage cell line RAW 264.7 was stimulated by LPS (50 ng/ml) to induce iNOS expression (4, 6). 48 h later, the cells were incubated with lactacystin (40 μM for 20 h) in experiments similar to those described above for RT4 cells. Lactacystin blocked murine iNOS degradation, leading to its accumulation as detected by Western analysis (Fig. 9A) and by increased iNOS activity, evaluated by measuring nitrite accumulation in culture media (Fig. 9B). These results indicate that targeting iNOS to the proteasome occurs in both human and murine cells, and it occurs independent of the mechanism of iNOS induction, e.g. by transfection (HEK293), cytokines (RT4), or LPS (RAW 264.7).

Traditionally, it has been thought that all substrates of the proteasome pathway must be ubiquitinated as a prelude to their destruction. Recently, however, there have been several studies showing examples of proteins that are degraded by proteasomes independent of ubiquitination. Moreover, as it has been demonstrated with p21, ubiquitination of a protein is no longer sufficient to conclude that its degradation must proceed through a ubiquitinated intermediate (28, 29). In our study, it is not clear if the accumulated iNOS after proteasomal inhibition is ubiquitinated and whether the ubiquitination of iNOS is required for its degradation. Higher molecular masses of proteins on immunoblots are often interpreted to be caused by mult ubiquitin conjugation (28, 29). Higher molecular complexes of iNOS were observed in SDS/polyacrylamide gel electrophoresis immunoblots, particularly after proteasomal inhibition (see Figs. 1, 2, 5, and 6). The identity of these complexes, however, remains to be elucidated.

It has become clear that cells control the level of their gene

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**Fig. 8.** The proteasome inhibitor lactacystin blocks degradation of cytokine-induced human iNOS in RT4 cells. RT4 cells were incubated for 24 h in the presence of the same cytokine mixture as described in the Fig. 7 legend, followed by a 24-h incubation with a fresh culture medium without cytokines and then in the presence of vehicle only or 20–40 μM lactacystin for an additional 20 h. The cell lysates (50 μg) were evaluated by Western blotting using an anti-iNOS antibody. Note that after iNOS induction by cytokines, the addition of lactacystin blocked iNOS degradation.

**Fig. 9.** The proteasome inhibitor lactacystin blocks degradation of LPS-induced murine iNOS in RAW 264.7 cells. Cells from the murine macrophage cell line RAW 264.7 were incubated for 24 h in the presence of 50 ng/ml LPS, followed by a 24-h incubation with a fresh culture medium without LPS and then in the presence of vehicle only or 40 μM lactacystin for an additional 20 h. A, the cell lysates (40 μg) were evaluated by Western blotting using an anti-iNOS antibody. B, nitrite accumulation (20 h) in the culture medium is expressed as nanomoles/milligram of total cell protein (mean ± SD). Note that after iNOS induction by LPS, the addition of lactacystin blocked iNOS degradation.
expression, in part through their control over protein degradation. Understanding the regulation of iNOS degradation will reveal how cells control the level of NO synthesis during inflammation and host defense. For instance, it has already been shown that, in activated mouse peritoneal macrophages, enhancement of iNOS degradation partially contributes to the mechanisms of suppression of NO release by transforming growth factor-β (30). Recently, the irreversible inactivator of neuronal NOS guanabenz has been shown to enhance the proteolytic turn over of the enzyme by a mechanism involving the proteasome (31). Finally, while this study was being reviewed, Felley-Bosco et al. (32) reported that in human colon carcinoma cells, caveolin-1 down-regulates iNOS via the proteasome pathway.

Potentially, the acceleration of iNOS degradation may prove to be an efficient approach for NO modulation, because the process of targeting cellular proteins for degradation is highly selective (11–13). By characterizing the specific pathway for iNOS degradation, our study lays the groundwork for such endeavors.

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