Inducible Nitric Oxide Synthase is Regulated by the Proteasome Degradation Pathway

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Running Title: iNOS degradation by the Proteasome Pathway

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SUMMARY

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 ALLN resulted in 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 L-NAME. Furthermore, proteasomal inhibition blocked degradation of an iNOS splice variant that lacked 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 calpastain, and lysosomal inhibitors, E64, leupeptin, pepstatin A, chloroquine and NH₄Cl 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 IκB-α, thus preventing activation of NF-κB. In contrast, lactacystin, added 48 h following iNOS induction, led to accumulation of iNOS. Similarly, in murine macrophage cell line RAW 264.7, lactacystin blocked iNOS degradation, when added 48 h following iNOS induction by LPS. These data identify the proteasome as the primary degradation pathway for iNOS.
**Introduction**

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 calcium (Ca\(^{2+}\))-dependent isoforms, neuronal NOS and endothelial NOS (or NOS1 and NOSIII, respectively). Ca\(^{2+}\)-activated calmodulin binds to and transiently activates constitutive NOS dimers (2, 3). Due to 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 whose activity 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. Lactacytin, calpastatin, taurocholic acid were obtained from Calbiochem-Novabiochem. N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN), N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norleucinal (MG132), trans-epoxysuccinyl-L-leucylamido-4-guanidino butane (E64), leupeptin, pepstatin-A, chloroquine, NH₄Cl, N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and lipopolysaccharide (LPS) were from Sigma. All other reagents were purchased either from Fisher Scientific or from sources stated in the text.

Antibodies. 1E8-B8 (Research and Diagnostic Antibodies) is a monoclonal antibody specific for the iNOS isoform and can detect both human and murine iNOS (8, 14). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Advanced Immunochemical. A goat polyclonal antibody (Santa Cruz) and a rabbit polyclonal antibody (New England Biolabs) were used in immunoblotting for detection of IκB-α and phosphorylated IκB-α, respectively.

Cell Culture. Human embryonic kidney (HEK) 293, RT4 (human bladder transitional cell papilloma) and murine macrophage RAW 264.7 cell lines were purchased from American Type Culture Collection. Cells were cultured in phenol-red free IMEM medium (HEK293), DMEM medium (RAW 264.7), or McCoy’s medium (RT4), supplemented with 2 mM glutamine, and 10% heat-inactivated, filtered (40 nm-filter) fetal bovine serum (Hyclone Laboratories, Inc.) at 37ºC in 5% CO₂.

Transfections. Human iNOS cDNA, inserted into the expression vector pRc/CMV (Invitrogen Corp.) under the control of the cytomegalovirus promoter, was used. Cationic lipid-mediated transient transfection was done using "Lipofectamine" and a transfection enhancing "Plus reagent" (Life Technologies Inc.) following manufacturer’s instructions.

iNOS Induction in RT4 Cells and RAW 264.7. RT4 cells were incubated with or without a mixture of interleukin-1β (IL-1β, 0.5 ng/ml), tumor necrosis factor-α (TNF-α, 10 ng/ml), and
interleukin-6 (IL-6, 200 units/ml) (6, 15). RAW 264.7 were incubated with or without 50 ng/ml LPS (4, 6).

**Analysis of iNOS Protein Levels.** After gentle rinsing twice with PBS, the cell layer was lysed on ice for 45 min, in iNOS extraction buffer of 40 mM Bis-Tris propane buffer, pH 7.7, 150 mM NaCl, 10% glycerol with 20 mM of the detergent sodium taurocholate and in the presence of protease inhibitors [PMSF (1 mM), pepstatin A (10 µg/ml), leupeptin (10 µg/ml), aprotinin (10 µg/ml), phenanthroline (10 µg/ml), and benzamidine HCl (16 µg/ml)] (PharMingen). Lysates were centrifuged (16,000 X g, 5 min, 4°C) and supernatants stored at -80°C (14). In some experiments, in order to lyse cells and precipitate proteins immediately, 20% trichloroacetic acid or 1% boiling SDS was added directly to cells. Total protein concentrations were determined by bicinchoninic acid reagent (Pierce). Cell lysates (50 µg) were mixed with one third volume of 4X Laemmli sample buffer (200 mM Tris-HCl, pH 6.8/ 8% SDS/ 0.004% bromophenol blue/ 40% glycerol/ 400 mM DTT), boiled at 95°C for 5 min and then subjected to SDS/polyacrylamide gel electrophoresis (PAGE). Following SDS/PAGE, immunoblotting was done with 1E8-B8 antibody. An enhanced chemiluminescence system was used for detection (SuperSignal West Pico; Pierce). Images were acquired using a cooled charged couple device camera (Eagle Eye II Still Video System; Stratagene).

**NOS Activity.** NOS activity in cell culture was determined by measuring the accumulation of nitrite in culture media. 100 µl of culture medium was mixed with 100 µl of Griess reagent for 10 min at room temperature and absorbance at 543 nm was recorded in a microplate reader. Serial dilutions of sodium nitrite were used as standards (16).
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 NOS genes and has been extensively used to study exogenously expressed 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 non-specific proteasome inhibitors, ALLN and MG132, and of 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, which could be detected by immunoblotting of cell lysates with specific antibody for iNOS (Fig. 1A). In some experiments, in order to lyse cells and precipitate proteins immediately, 20% trichloroacetic acid (Fig. 1B) or boiling SDS containing buffer (Fig. 1C) was added directly to the cells. In all cases, inhibition of proteasomal activity with lactacystin led to iNOS accumulation, indicating that the effect of proteasomal inhibition on iNOS was not the result of *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 HEK293-iNOS cells for 20 hr with increasing concentrations of lactacystin (1-20 μM) resulted in corresponding increase in iNOS accumulation (Fig. 2). In similar experiments, incubation of HEK293-iNOS cells with as little as 2 μM of 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 following lactacystin addition and continues 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 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 or not the proteasomal degradation of human iNOS is dependent 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, $N^\omega$-nitro-L-arginine methyl ester (L-NAME) (19). Incubation of cells in the presence of 3 mM L-NAME resulted in inhibition of NO synthesis by iNOS as estimated by measuring nitrite accumulation in culture media (data not shown). Incubation of cells with 10 $\mu$M 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 $\mu$M) 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, Respectively.** Like all NOS isoforms, for the synthesis of NO, 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$_{8-9}$) a domain critical for dimerization and NO synthesis (14). Therefore iNOS$_{8-9}$ can not form dimers nor produce NO. In addition, we have recently identified Asp 280 in human iNOS as a residue critical for proper subunit interaction and substrate binding$^2$. iNOS$_{D280A}$ is a mutant of human iNOS in which Asp 280 is replaced by Ala. Although iNOS$_{D280A}$ retains its ability to dimerize, it can not bind the substrate L-arginine and hence it represents an inactive dimer form of iNOS (21). To establish if these iNOS variants are degraded via the proteasome pathway, we transfected HEK293 cells with cDNAs encoding wild-type human iNOS, iNOS$_{8-9}$, or iNOS$_{D280A}$ and examined the effect of proteasome inhibition. Similar to the effect seen on wild-type iNOS, lactacystin blocked the degradation of iNOS$_{8-9}$, and of iNOS$_{D280A}$ (Fig. 5). These results suggest that targeting of human iNOS by the proteasome degradation pathway is independent of the capacity of iNOS to dimerize or to 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. Following 20 h incubation in the presence of the specific inhibitor, cells were immediately lysed by boiling in SDS-containing buffer. In contrast to proteasomal inhibitors, the calpain inhibitor calpastatin (5 µM), inhibitor of the trypsin-like proteases TLCK (100 µM), or inhibitor of the chymotrypsin-like proteases TPCK (50 µM) did not lead to accumulation of iNOS (Fig. 6A). Similar results were obtained using the lysosomal protease inhibitors E64 (50 µM), leupeptin (50 µM), the aspartate protease inhibitor pepstatin-A (100 µM), the lysosomal acidification inhibitors chloroquine (100 µM), or NH₄Cl (20 mM) (Fig. 6 B and C). Yet, there was mild but not consistent increase iNOS detection with lysosomal inhibitors (Fig. 6). Because of the mild increase seen in some of the iNOS immunoblot signals in figure 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: Lactacystin Prevents iNOS induction by cytokines. NF-κB 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-κB 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 IκBα. Therefore, proteasomal inhibitors block the degradation of already phosphorylated and ubiquitinated IκBα, thus aborting NF-κB activation (25). Inhibitors of the proteasome pathway were found to interfere with the induction of iNOS in rat alveolar macrophages by blocking NF-κB activation (26). We hypothesized that the proteasome regulates iNOS on two distinct levels; at the transcriptional level, by degrading IκBα and thus
activating NF-κB, and at the posttranslational level by degrading iNOS protein. To test this hypothesis we used a cytokine mixture of IL-1β (0.5 ng/ml), TNF-α (10 ng/ml) and IL-6 (200 u/ml) to induce iNOS expression in RT4 cells (6, 15). Addition of 40 µM lactacystin 1 h prior to stimulation prevented iNOS induction (Fig. 7A). The inhibitory effect on iNOS induction was due to inhibition of IκBα degradation, as suggested by increased levels of IκBα (Fig. 7B). The increase in IκBα seen with proteasomal inhibition was due to inhibition of the degradation of already phosphorylated IκBα as demonstrated by immunoblotting cell lysates with anti phospho-IκBα 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 24 h incubation with fresh medium without cytokines and then in the presence of lactacystin (20-40 µM for 20 h). Treatment of cells with lactacystin led to a dose-dependent accumulation of iNOS (Fig. 8), similar to that seen in experiments with transfected HEK293 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 indicate 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). Forty-eight hours later, 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 an ubiquitinated intermediate (28, 29). In our study, it is not clear if the accumulated iNOS following proteasomal inhibition is ubiquitinated and whether or not ubiquitination of iNOS is required for its degradation. Higher molecular masses of proteins on immunoblots are often interpreted to be due to multiubiquitin conjugation (28, 29). Higher molecular complexes of iNOS were observed in SDS/PAGE immunoblots, particularly following proteasomal inhibition (see figures 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 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 been already 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, Fell-Bosco et al. reported that, in human colon carcinoma cells, caveolin-1 down-regulates iNOS via the proteasome pathway (32).

Potentially, acceleration of iNOS degradation may prove to be an efficient approach for NO modulation since 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.

Acknowledgments - This study is dedicated to the memory of Joseph R. Rodarte, a mentor and a friend. His life continues through the students he mentored and the souls he touched. Thank you Joe. The work was supported by The American Lung Association, Caroline Wiess Law Fund for Molecular Medicine, The Methodist Foundation and by a T.T. Chao Scholar Award.

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Footnotes

1 The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; iNOS\(_{\text{wt}}\), wild-type iNOS; iNOS\(_{8-9-}\), iNOS splice variant with deletion of exons 8 and 9; HEK293, Human embryonic kidney cell line; HEK293-iNOS, HEK293 cell line stably expressing iNOS; RT4, human bladder transitional cell papilloma; H\(_4\)B, (6R)-5,6,7,8-tetrahydro-L-biopterin; Ca\(^{2+}\), calcium; L-NAME, N\(^{\text{a}}\)-nitro-L-arginine methyl ester; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); ALLN, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; MG132, N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norleucinal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TLCK, N-\(\alpha\)-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-\(p\)-tosyl-L-phenylalanine chloromethyl ketone; E64, trans-epoxysuccinyl-L-leucylamido-4-guanidino butane; LPS, lipopolysaccharide.

2 Manuscript in preparation
Figure Legends

**FIG. 1. The proteasome inhibitors, lactacystin, ALLN and MG132 block human iNOS degradation.** HEK293 cells, stably expressing human iNOS, were incubated for 20 h in the presence of 10 µM lactacystin, 100 µM ALLN, or 10 µM MG132. In parallel experiments, cells were incubated in the presence of vehicles used to dissolve the corresponding inhibitors. Cells were harvested by lysis on ice for 45 min, in 40 mM Bis-Tris propane buffer, pH 7.7, 150 mM NaCl, 10% glycerol with 20 mM sodium taurocholate (A). In some experiments, in order to lyse cells and precipitate proteins immediately, 20% trichloroacetic acid (B) or 1% boiling SDS (C) was added directly to cells. Cell lysates (50 µg) were subjected to SDS/PAGE followed by immunoblotting with anti-human iNOS antibody. The results are representative of three independent experiments performed in duplicate.

**FIG. 2. The specific proteasome inhibitor, lactacystin blocks human iNOS degradation in a dose dependent manner.** HEK293 cells, stably expressing human iNOS, were incubated for 20 h in the presence of vehicle only or 1-20 µM lactacystin. Cell lysates (50 µg) were evaluated by Western blotting using anti-iNOS antibody. The data are representative of three independent experiments performed in duplicate.

**FIG. 3. Time Course for iNOS accumulation following proteasomal inhibition with lactacystin.** (A) HEK293 cells, stably expressing human iNOS, were incubated in the presence of vehicle only or 2 µM lactacystin for 8-32 h. Cell lysates (50 µg) were evaluated by Western blotting using anti-iNOS antibody. The data are representative of three independent experiments performed in duplicate. (B) Quantification of iNOS signal in A, expressed as the percentage increase in lactacystin treated cells compared to cells treated with vehicle only.

**FIG. 4. Lactacystin blocks human iNOS degradation notwithstanding NO production by iNOS.** HEK293 cells, stably expressing human iNOS, were incubated for 20 h in the presence of vehicle only or 10 µM lactacystin. Incubation of cells was done in the presence or absence of the NOS inhibitor L-NAME. Cell lysates (50 µg) were evaluated by Western blotting.
using anti-iNOS antibody (A) or with an antibody against GAPDH (B). The data are representative of two independent experiments performed in duplicate.

**FIG. 5. Lactacystin blocks degradation of a splice variant (iNOS<sub>8-9-</sub>) and a mutant (iNOS<sub>D280A</sub>) of human iNOS that lack the capacity for dimerization or substrate binding, respectively.** HEK293 cells were transfected with plasmids containing cDNA of human iNOS<sub>wt</sub>, iNOS splice variant iNOS<sub>8-9-</sub> or iNOS mutant with Ala replacing Asp in position 280 (iNOS<sub>D280A</sub>). Forty-eight hours after transfection, cells were incubated for 20 h in the presence or absence of 10 µM lactacystin. Cell lysates (50 µg) were evaluated by Western blotting using anti-iNOS antibody. The data are representative of three independent experiments.

**FIG. 6. Inhibitors of calpains, trypsin-like proteases and chymotrypsin-like proteases and lysosomal inhibitors do not block human iNOS degradation.** HEK293 cells, stably expressing human iNOS, were incubated for 20 h in the presence of 5 µM calpastatin, 100 µM TLCK, 50 µM TPCK (A), 50 µM E64 (B), 50 µM leupeptin, 100 µM pepstatin A, 100 µM chloroquine, or 20 mM NH<sub>4</sub>Cl (C). In parallel experiments cells were incubated in the presence of vehicle only or 10 µM of the proteasome inhibitor lactacystin. Cells were lysed by immediate boiling in 1% SDS-containing buffer and their lysates (50 µg) were evaluated by Western blotting using an antibody against human iNOS. The data are representative of three independent experiments performed in duplicate.

**FIG. 7. Proteasomal inhibition prevents cytokine induction of iNOS by blocking NFκB activation.** RT4 cells were incubated for 1 h in the presence of vehicle only or 40 µM lactacystin followed by a 24 h-incubation with a fresh culture medium in the presence of a cytokine mixture of IL-1β (0.5 ng/ml), TNF-α (10 ng/ml) and IL-6 (200 u/ml). Cell lysates (50 µg) were evaluated by Western blotting using an antibody against iNOS (A; 24h following cytokine induction), anti-IκB-α antibody (B; 1 h following cytokine induction), or anti-Phospho-IκB-α antibody (C; 1 h following cytokine induction). Note that proteasomal inhibition by lactacystin prevented the induction by cytokines of iNOS.
**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 in figure 7, 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 of lactacystin for an additional 20 h. Cell lysates (50 µg) were evaluated by Western blotting using anti-iNOS antibody. Note that following iNOS induction by cytokines, addition of lactacystin blocked iNOS degradation.

**FIG. 9.** The proteasome inhibitor lactacystin blocks degradation of LPS-induced murine iNOS in RAW 264.7 cells. 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 of lactacystin for an additional 20 h. (A) Cell lysates (40 µg) were evaluated by Western blotting using anti-iNOS antibody. (B) Nitrite accumulation (20 h) in the culture medium is expressed as nanomoles per milligram of total cell protein (mean ± SD). Note that following iNOS induction by LPS, addition of lactacystin blocked iNOS degradation.
Figure 1.
Figure 2.

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Figure 3.

A.

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Lactacystin: - + - + - + - + +

B.

% Increase in iNOS Signal

Time (h)

- 5
- 10
- 15
- 20
- 25
- 30
- 35

% Increase: 0 50 100 150 200
Figure 4.

A.

kDa

215 -
132 -
95 -

L-NAME

- - + +

Lactacystin

- + - +

B.

kDa

42 -
31 -

← iNOS

← GAPDH
Figure 5.
Figure 7.

A. 
- **kDa**
  - 132
- **Vehicle**
- **Lactacystin**
  - iNOS

B. 
- **kDa**
  - 43
  - 33
- **IκB-α**
  - Phospho-IκB-α

C. 
- **kDa**
  - 43
  - 33
- **Cytokines**
  - +
  - +
Figure 8.
Figure 9.

A. 

B.

Nitrite in Medium (nmol/mg)
Inducible nitric oxide synthase is regulated by the proteasome degradation pathway
Aleksandra Musial and N. Tony Eissa

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