Ubiquitination of inducible nitric oxide synthase is required for its degradation

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Inducible nitric oxide synthase (iNOS) is responsible for nitric oxide (NO) synthesis from L-arginine in response to inflammatory mediators. We have previously shown that iNOS is degraded through the 26S proteasome. Targeting of proteins for proteasomal degradation may or may not require their covalent linkage to multiubiquitin chains (ubiquitination). In addition, ubiquitination of a protein can serve functions other than signaling proteolysis. In this context, it is not known whether iNOS is subject to ubiquitination or whether ubiquitination is required for its degradation. In this study, we show that iNOS, expressed in HEK293 cells or induced in primary bronchial epithelial cells, A549 cells, or murine macrophages, is subject to ubiquitination. To investigate whether iNOS ubiquitination is required for its degradation, HEK293T cells were cotransfected with plasmids containing cDNAs of human iNOS and of the dominant negative ubiquitin mutant K48R. Disruption of ubiquitination by K48R ubiquitin resulted in inhibition of iNOS degradation. ts20 is a mutant cell line that contains a thermolabile ubiquitin-activating enzyme (E1) that is inactivated at elevated temperature, preventing ubiquitination. Incubation of ts20 cells, stably expressing human iNOS, at the nonpermissive temperature (40°C) resulted in inhibition of iNOS degradation and marked accumulation of iNOS. These studies indicate that iNOS is subject to ubiquitination and that ubiquitination is required for its degradation.

Nitric oxide (NO), an important signaling and cytotoxic molecule, is synthesized from L-arginine by isoforms of nitric oxide synthase (NOS; refs. 1–3). 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 (1, 2). 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 (3, 4). 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 (5). 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 (6, 7). 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 (8).

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 the mechanisms of its degradation. Recently, the 26S proteasome has been identified as the major pathway responsible for iNOS degradation (9, 10). However, the specific mechanisms underlying how iNOS is targeted for proteasomal degradation remain to be elucidated.

Targeting of cellular proteins for proteasomal proteolysis is a highly complex, and tightly regulated process (11). Ubiquitin, an evolutionarily conserved protein of 76 residues, has diverse cellular functions, including marking proteins for degradation by the proteasomal or the lysosomal pathway. However, degradation through the proteasome pathway also includes many nonubiquitinated proteins (11–13). In addition, some proteins that undergo ubiquitination do not require that ubiquitination before undergoing proteasomal degradation (13, 14). Ubiquitination, in these proteins, may serve other functions rather than signaling proteolysis (11, 13, 15). Thus, ubiquitination of a protein is insufficient to conclude that the protein degradation must proceed through a ubiquitinated intermediate (13). In the context of iNOS degradation, it is not known whether iNOS is ubiquitinated and whether or not ubiquitination of iNOS is required for its degradation. In this regard, this study addresses these two key questions. Resolving these questions is a required cornerstone to efforts aiming at dissecting the molecular machinery of iNOS degradation. Understanding this machinery is a prerequisite for therapeutic strategies aimed at modulating NO synthesis by iNOS.

Materials and Methods

Reagents. *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norleucinal (MG132), *N*-ethylmaleimide (NEM), lipopolysaccharide, and Triton X-100 were from Sigma. Tween 20, prestained molecular mass standards were from Bio-Rad. All other reagents were purchased from Fisher or sources stated in the text.

Antibodies. Monoclonal antibodies 1E8-B8 and 21C10-D10 (Research and Diagnostic Antibodies, Benicia, CA) and polyclonal antibodies 06-295 and 06-573 (Upstate Biotechnology, Lake Placid, NY) are specific for iNOS isoform. U5379 (Sigma) and Z0458 (Dako) are both polyclonal antiubiquitin antibodies. 1E8-B8 and U5379 antibodies were used for Western analyses for iNOS and ubiquitin, respectively. 12CA5 (Roche) is a monoclonal antibody against hemagglutinin (HA) of human influenza virus. MAB3520 (Chemicon) is a monoclonal antibody against ubiquitin activation enzyme E1. 6C6 (Advanced Immuno-Chemical, Long Beach, CA) is a monoclonal antibody against GAPDH. IκB-β C-20 (Santa Cruz Biotechnology) is a polyclonal antibody against IκB-β.

Cell Culture. Human embryonic kidney (HEK) 293, A549 (human alveolar type II epithelium-like lung carcinoma cell line), and murine macrophage RAW 264.7 cell lines were purchased from American Type Culture Collection (ATCC). HEK293T, a HEK293 cell line that expresses large T antigen, was provided by

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Abbreviations: iNOS, inducible nitric oxide synthase; HEK, human embryonic kidney; MG132, *N*-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norleucinal; NEM, *N*-ethylmaleimide; HA, hemagglutinin; TNF, tumor necrosis factor.

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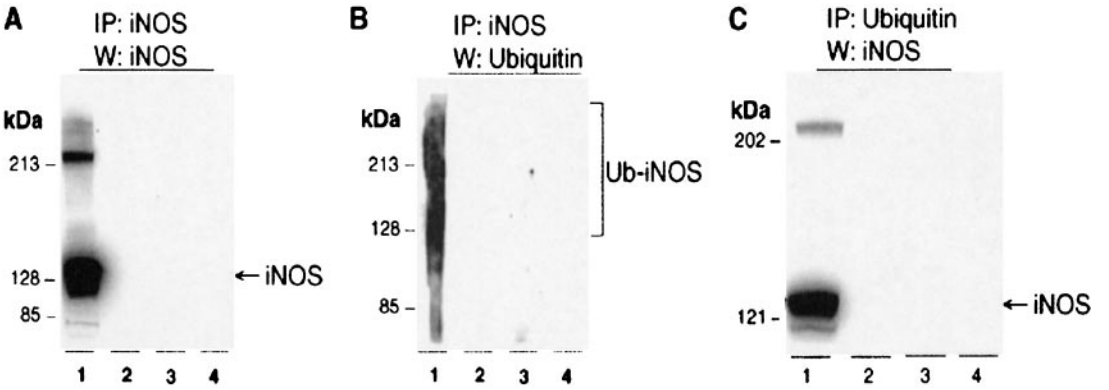

Fig. 1. Human iNOS is subject to ubiquitination in HEK293 cells. HEK293 cells, stably expressing human iNOS, were harvested by lysis on ice for 30 min, in 40 mM Bis-Tris propane buffer (pH 7.7), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 5 mM NEM. Cell lysates were subjected to immunoprecipitation (IP) with anti-iNOS (21C10-D10) antibody (lane 1 in *A* and *B*) or with anti-ubiquitin (Z0458) antibody (lane 1 in *C*). An aliquot of the immunoprecipitate was subjected to Western blotting (W) with anti-iNOS antibody (*A* and *C*) or with anti-ubiquitin antibody (*B*). To verify the stringency of immunoprecipitation, experiments included samples where immunoprecipitating antibody was omitted (lane 2) or replaced with irrelevant antibody (anti-IkB-β, lane 3), or immunoprecipitation was done on lysates of HEK293 cells that do not express iNOS (lane 4). Note that the high molecular mass complexes detected in lane 1 in *B* represent ubiquitinated iNOS (Ub-iNOS).

L. Feng (Baylor College of Medicine). Mutant ts20 and their wild-type counterpart E36, both derived from a Chinese hamster lung cell line (16), were provided by G. Strous at Utrecht University, The Netherlands. The mutant ts20 cell lines has a thermolabile ubiquitin-activating enzyme E1 that is irreversibly inactivated at the nonpermissive temperature 40°C, leading to disruption of ubiquitination (16–19). HEK 293 and HEK293T cells were cultured in improved MEM (Biofluids, Rockville, MD), A549 in F-12K medium (ATCC), RAW 264.7 in DMEM with high glucose (Biofluids), and both E36 and ts20 in minimum essential medium-α (Invitrogen). Each medium was supplemented with 2 mM glutamine, and 10% heat-inactivated FBS (HyClone Laboratories). All cells were maintained in 5% CO_2 and at 37°C, except that E36 and ts20 cells were maintained at 30°C (the permissive temperature for ts20 E1 enzyme).

Primary Bronchial Epithelial Cells. Normal human tracheobronchial epithelial cells, purchased from Clonetics (San Diego), were subcultured, and passage-2 cells were cultured at the air/liquid interface at a density of 1×10^5 cells onto 24-mm-diameter semipermeable membrane inserts (Transwell-clear; Corning) in serum-free, 1:1 mixture of DMEM (Invitrogen) and bronchial epithelium growth medium (BEGM; Clonetics), containing insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), epidermal growth factor (0.5 ng/ml), BSA (0.5 mg/ml), bovine pituitary extract (1% vol/vol), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and retinoic acid (5×10^{-8} M) as described (20–22). The cultures were grown submerged for the first 7 days, at which time the air/liquid interface was created by removing media from the apical compartment of the cultures. The culture media were changed every other day until the air/liquid interface was created. Thereafter, culture media were changed daily by replacing fresh media only to the basal compartment. Cultures were maintained at 37°C in a humidified 5% CO_2 for further 4 wk after confluence until they reach fully differentiated mucociliary plateau phase. The early passage human tracheobronchial epithelial cells cultured at the air/liquid interface preserve the ability of these cells to differentiate into mucous and ciliated cells. The morphologic pattern of their differentiation is similar to that observed *in vivo* (22).

Transfection and Stable Cell Lines Production. Cationic lipid-mediated transient transfection was done by using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. Stable cell lines of HEK293, ts20, and E36 cells, expressing iNOS, were produced by using transfection of iNOS cDNA followed by positive colony selection using G418 (Invitrogen) at concentration of 600 μg/ml (HEK293) or 450 μg/ml (ts20 and E36) (23, 24).

Cell Lysis. After gently rinsing twice with PBS, the cell layer was lysed on ice for 30 min in 40 mM Bis-Tris propane buffer (pH 7.7), 150 mM NaCl, 10% glycerol, and 1% Triton X-100 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), benzamidine HCl (16 μg/ml); PharMingen], and 5 mM NEM. Lysates were centrifuged (16,000 $\times$ *g*, 5 min, 4°C), and supernatants were stored at −80°C. Total protein concentrations were determined by using a bicinchoninic acid reagent, following the manufacturer's instructions (Pierce).

Immunoprecipitation. To immunoprecipitate iNOS, cell lysates (0.5 mg) were incubated at 4°C with anti-iNOS antibody in a total volume of 500 μl of lysis buffer for 90 min and then with rabbit polyclonal anti-mouse antibody for 30 min. Protein A-Sepharose beads (50 μl of 10% solution; Pharmacia) were added to the samples. After further incubation for 1 h at 4°C, beads were washed three times (1 ml each) in ice-cold lysis buffer. Immunoprecipitated proteins were eluted by heating at 95°C for 5 min in Laemmli sample buffer (50 mM Tris·HCl, pH 6.8/2% SDS/0.001% bromophenol blue/10% glycerol/100 mM dithioerithreitol; ref. 25). To immunoprecipitate ubiquitin conjugates, a similar procedure was followed, except that a polyclonal anti-ubiquitin antibody was used.

Western Analysis. Cell lysates (50 μg) or 28 μl of immunoprecipitated proteins were mixed with one third volume of 4× Laemmli sample buffer (200 mM Tris·HCl, pH 6.8/8% SDS/0.004% bromophenol blue/40% glycerol/400 mM DTT), heated at 95°C for 5 min. Proteins were resolved on SDS/PAGE (NOVEX, San Diego) at 125 V and transferred to nitrocellulose membranes for 1 h at 20 V by using semidry transfer cell (Bio-Rad). When probing with antiubiquitin antibody, blot membranes, after transfer of electrophoresed proteins, were autoclaved at 121°C in distilled water for 20 min to ensure complete denaturation of ubiquitinated proteins and to expose any masked ubiquitin epitopes (26). Immunoreactive bands were visualized with the use of enhanced chemiluminescence system (SuperSignal West Pico, Pierce). Images were acquired by using

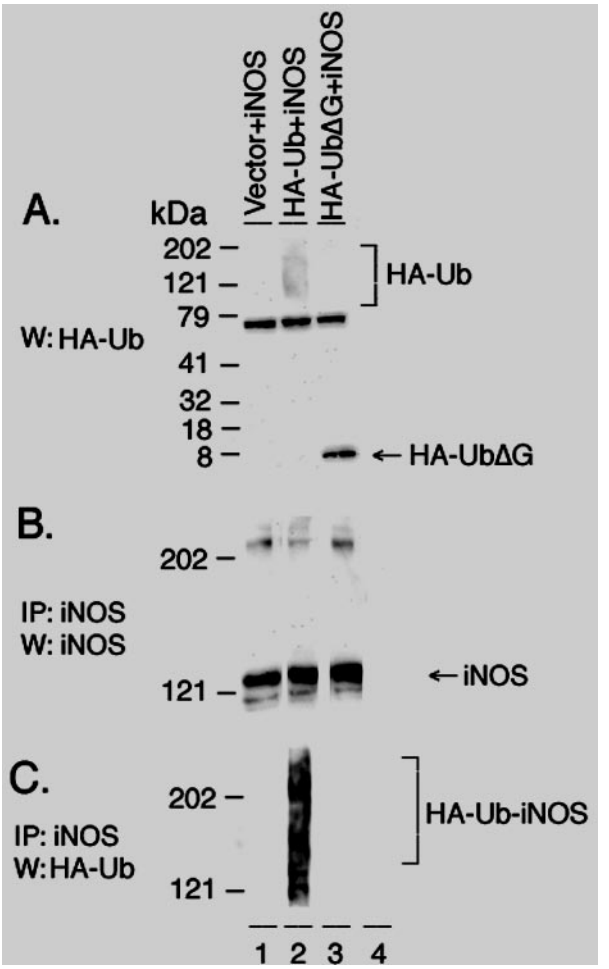

Fig. 2. Cotransfection of HEK 293T cells with human iNOS and exogenous ubiquitin results in iNOS ubiquitination. HEK293T cells were cotransfected with plasmids containing cDNA of human iNOS and of either an empty vector (lane 1), HA-tagged ubiquitin (HA-Ub; lane 2), or an HA-tagged ubiquitin mutant (HA-UbΔG; lane 3) that cannot ubiquitinate substrates. The molar ratio of iNOS plasmid to the coplasmid was 1:10, respectively. Thirty-six hours after transfection, cells were lysed as described for Fig. 1. An aliquot of cell lysates (50 μg) was analyzed by Western blot with anti-HA antibody (*A*). Note that the high molecular mass complexes detected in lane 2 represent HA-Ub linked to ubiquitinated proteins. HA-UbΔG cannot ubiquitinate proteins and was detected as a single band of ≈8.6 kDa in lane 3. The ≈75-kDa band is due to nonspecific binding of HA antibody because it was also seen with nontransfected cells (data not shown). In addition, cell lysates were subjected to immunoprecipitation with anti-iNOS antibody. Immunoprecipitates were subjected to Western analysis with anti-iNOS antibody (*B*) or anti-HA antibody (*C*). Cotransfection of HA-Ub but not HA-UbΔG resulted in detection of the high molecular mass complexes (*C*, lane 2) that represent ubiquitinated iNOS (Ub-iNOS). To verify the stringency of immunoprecipitation, experiments included samples where immunoprecipitating antibody was omitted (lane 4 in *B* and *C*).

a cooled charge-coupled device camera (Eagle Eye II Still Video System, Stratagene).

Results and Discussion

Exogenously Expressed Human iNOS in HEK293 Cells Is Subject to Ubiquitination. We investigated the presence of ubiquitinated iNOS in HEK293 engineered to stably express human iNOS. iNOS was immunoprecipitated from HEK293 cell lysates with an iNOS-specific antibody, and the immunoprecipitates were evaluated for the presence of ubiquitinated iNOS. Ubiquitin-protein conjugates are known to be deubiquitinated by an NEM-sensitive pathway (11). To minimize deubiquitination of iNOS during cell lysis, lysis buffer included 5 mM of the ubiquitin isopeptidase inhibitor NEM (27, 28). The presence of iNOS in the immunoprecipitate was confirmed by immunoblotting with anti-iNOS antibody (Fig. 1*A*). No signals were detected in samples in which immunoprecipitating antibody was omitted or replaced with irrelevant antibody (anti-IkB-β), or in HEK293 cells that do not express iNOS, confirming both the specificity and the stringency of the immunoprecipitation. By using a ubiquitin-specific antibody for Western blot analysis, a high molecular mass smear, typical of ubiquitinated proteins (17, 19, 26, 29), was detected only in samples in which iNOS was present, indicating that they represent coimmunoprecipitated ubiquitinated iNOS (Fig. 1*B*). To further confirm the association between ubiquitin and iNOS in HEK293 cells, immunoprecipitation of ubiquitin-protein conjugates was performed on cell lysates by using an anti-ubiquitin antibody. Western analysis revealed the presence of iNOS in immunoprecipitated proteins (Fig. 1*C*). These data indicate that exogenously expressed human iNOS in HEK293 is subject to ubiquitination.

Proteasomal inhibitors such as MG132 and lactacystin are cell-permeable compounds that specifically block the activity of the 20S proteasome. Thus, they cause accumulation of ubiquitinated proteins that are degraded by proteasomes. Importantly, however, as shown in Fig. 1, ubiquitinated iNOS was clearly detectable even when cells were not pretreated with proteasomal inhibitors, suggesting that at least a portion of the ubiquitinated iNOS is not an immediate target for the proteasomal proteases (11, 27). When the experiments described in Fig. 1 were repeated in the presence of MG132 or lactacystin, there was an increase in detected ubiquitinated iNOS (data not shown).

Ubiquitination of Human iNOS in Cultured Cells Using Exogenous Ubiquitin. We sought to investigate whether we could ubiquitinate iNOS in HEK293 cells by using exogenously introduced ubiquitin. We cotransfected HEK293T cells with cDNAs encoding human iNOS and HA-tagged ubiquitin in a molar ratio of 1:10, respectively. In parallel experiments, HA-tagged ubiquitin (HA-Ub) was replaced with either empty vector or with an HA-tagged ubiquitin mutant (HA-UbΔG) in which the C-terminal Gly residue is deleted. In the first step of protein ubiquitination, ubiquitin is activated in its terminal Gly to a high-energy thiol ester intermediate, a reaction catalyzed by the ubiquitin-activating enzyme, E1 (11). Therefore, HA-UbΔG is not able to ubiquitinate proteins. HA-Ub transfected in HEK293T cells could be detected in cell lysates immunoblotted with anti-HA antibody as higher HA-Ub-protein conjugates (Fig. 2A). In contrast, in cells transfected with HA-UbΔG, higher HA-Ub-protein conjugates could not be detected, and HA-UbΔG migrated as a single band at ≈8.6 kDa, its expected size. Thirty-six hours after transfection, cell lysates were subjected to immunoprecipitation with anti-iNOS antibody. In cells transfected with iNOS, HA-Ub-iNOS conjugates were detected only when cells were cotransfected with HA-Ub but not when HA-UbΔG was used (Fig. 2 *B* and *C*).

Cytokine-Induced iNOS in Human Primary Bronchial Epithelial Cells Is Degraded by the Proteasome and Is Subject to Ubiquitination. In human lung, bronchial epithelial cells are the principal cell type responsible for iNOS production in response to cytokines and inflammatory mediators (4, 7). Up-regulation of iNOS in primary bronchial epithelial cells in asthma accounts for increased exhaled NO in these patients and has been implicated in the pathogenesis of airway inflammation, the hallmark of the disease (7). Therefore, it is important to understand the regulatory mechanisms of iNOS degradation in these cells. We have adopted a model of culturing primary bronchial epithelial cells in an air/liquid interface by using an *in vitro* culture system. In this system, primary cells differentiate to a heterogeneous

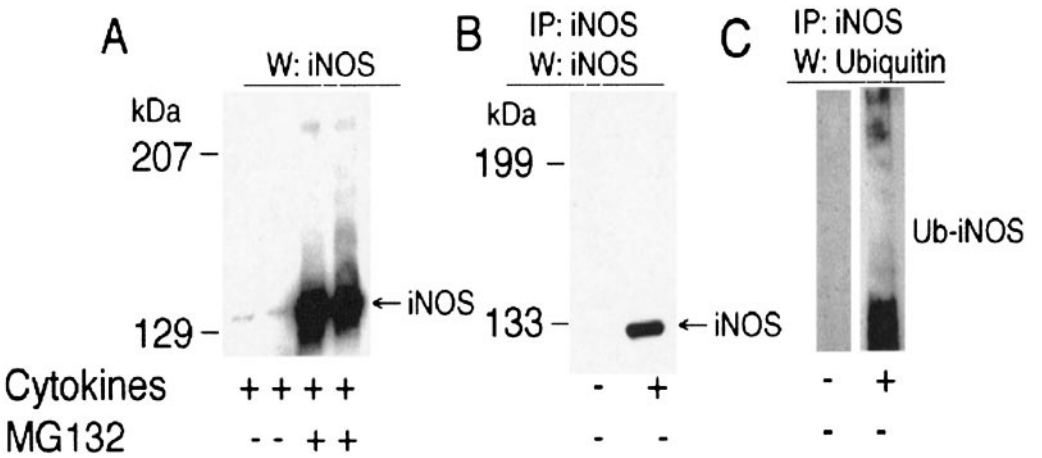

Fig. 3. iNOS is degraded by the proteasome and is subject to ubiquitination in human primary bronchial epithelial cells. (*A*) Primary cells were incubated for 24 h in the presence of cytokine mixture of IFN-γ (100 units/ml), IL-1β (0.5 ng/ml), and TNF-α (10 ng/ml) and IL-6 (200 units/ml). Cells were then incubated for 20 h in fresh culture medium containing either vehicle only (methanol) or 10 μM of the proteasome inhibitor MG132. Cell lysates were analyzed by Western blotting (W) with anti-iNOS antibody. In parallel experiments, primary cells stimulated with the same cytokine mixture for 24 h were lysed, and cell lysates were subjected to immunoprecipitation with anti iNOS (21C10-D10) antibody. An aliquot of the immunoprecipitate was subjected to Western blotting (W) with anti-iNOS antibody (*B*) or with anti-ubiquitin antibody (*C*).

population containing secretory, ciliated, and basal cells that mimic their *in vivo* appearance and function (20–22). We first sought to establish that, in primary cells, iNOS is degraded by the proteasome pathway. Fully differentiated primary bronchial epithelial cells were incubated for 24 h in the presence of cytokine mixture of IFN-γ (100 units/ml), IL-1β (0.5 ng/ml), and tumor necrosis factor (TNF)-α (10 ng/ml) and IL-6 (200 units/ml) (4, 7, 9). After cytokine induction of iNOS, addition of the proteasome inhibitor MG132 led to marked accumulation of iNOS (Fig. 3*A*). This finding indicates that iNOS is degraded by the proteasome pathway in primary bronchial epithelial cells. Coimmunoprecipitation experiments showed that, in primary cells, iNOS is subject to ubiquitination (Fig. 3 *B* and *C*). Similar results were obtained in A549 cells, a human alveolar type II epithelium-like lung cell line. A549 cells were incubated for 24 h in cytokine mixture of IFN-γ, IL-1β, and TNF-α. Coimmunoprecipitation of cell lysates with specific iNOS antibody, done on cell lysates, revealed the presence of iNOS-Ub conjugates (data not shown). The finding that cytokine-induced human iNOS in primary cells and in A549 cells is ubiquitinated indicates that

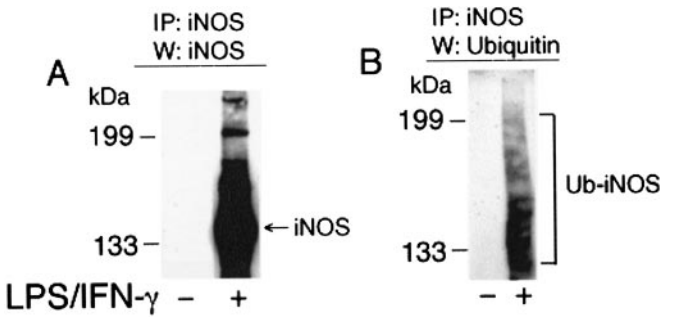

Fig. 4. Murine iNOS is subject to ubiquitination in RAW 264.7 cells. Cells from the murine macrophage cell line RAW 264.7 were incubated for 24 h with or without a mixture of lipopolysaccharide (LPS, 100 ng/ml) and the cytokine IFN-γ (10 u/ml). Cells were lysed as described in Fig. 1, and lysates were subjected to immunoprecipitation (IP) with anti-iNOS (06-573) antibody. An aliquot of the immunoprecipitate was subjected to Western blotting (W) with anti-iNOS antibody (*A*) or with anti-ubiquitin antibody (*B*). Note that the high molecular mass complexes detected in *B* represent ubiquitinated iNOS (Ub-iNOS).

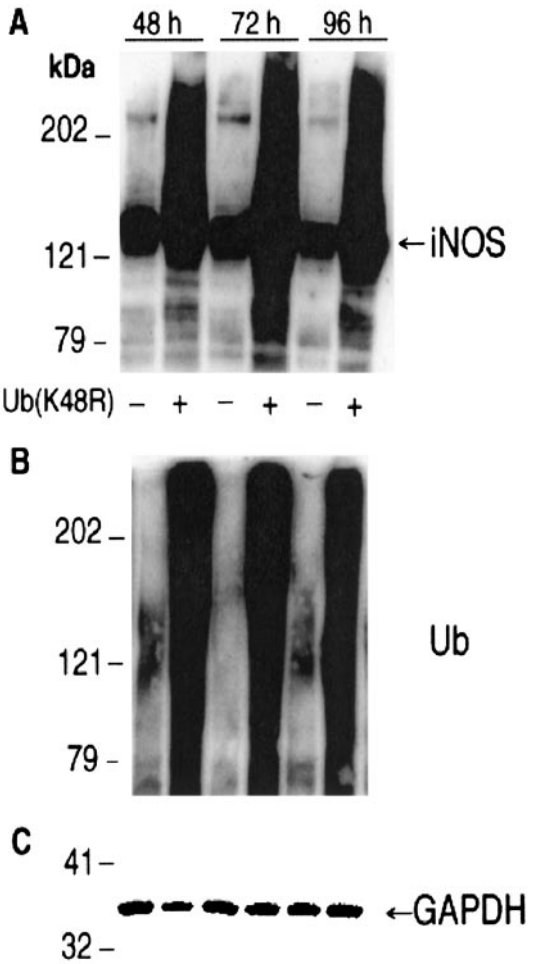

Fig. 5. Disruption of ubiquitination, by a dominant negative ubiquitin mutant leads to iNOS accumulation in HEK293T cells. HEK293T cells were cotransfected with plasmids containing cDNA of human iNOS and of either the ubiquitin mutant K48R or its empty vector. The molar ratio of iNOS plasmid to the coplasmid was 1:10, respectively. Cell lysates (50 μg) were subjected to SDS/PAGE followed by immunoblotting with antibodies against iNOS (*A*), ubiquitin (*B*), or GAPDH (*C*).

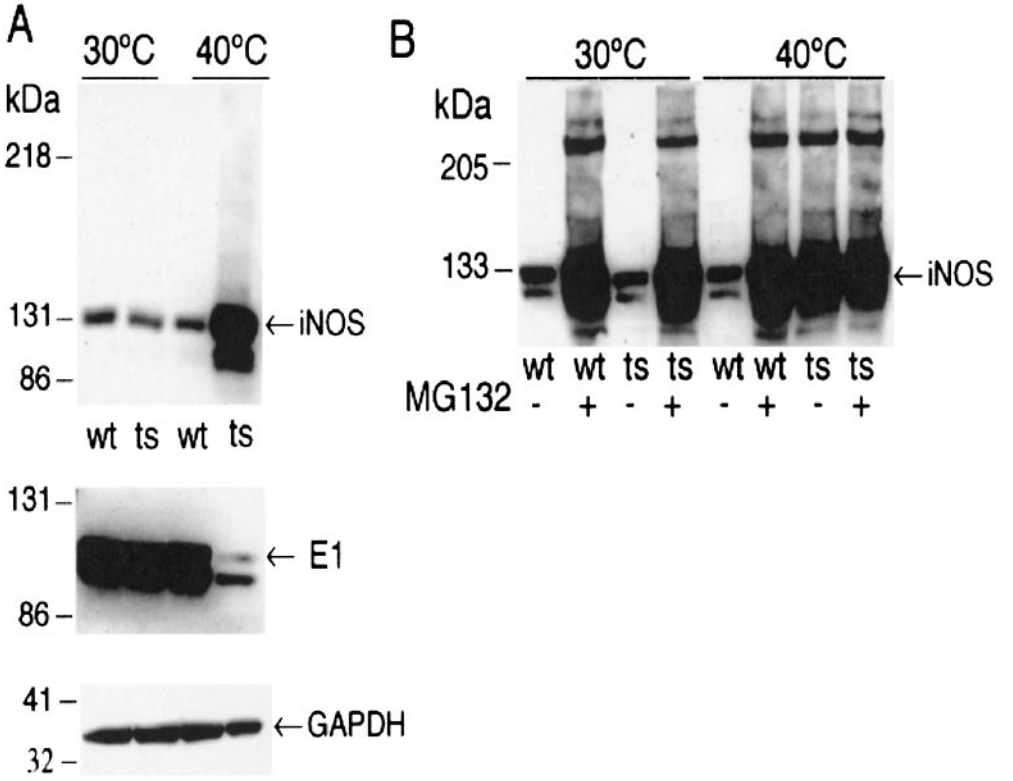

Fig. 6. Prevention of ubiquitination leads to iNOS accumulation in ts20 cells. (*A*) E36 (wt) and ts20 (ts) cells, stably expressing human iNOS, were incubated at the permissive temperature (30°C) or the nonpermissive temperature (40°C) for 24 h. Cell lysates were evaluated by Western blotting using antibodies against iNOS (*Top*), E1 (*Middle*), or GAPDH (*Bottom*). Note that incubation of ts20 cells at 40°C resulted in concomitant accumulation of iNOS and marked reduction of E1. Similar changes were absent in wild-type E36 cells. (*B*) Similar experiments were done as in *A* except cells were incubated in the presence of 10 μM of the proteasome inhibitor MG132 or its vehicle.

iNOS ubiquitination is not unique to HEK293 cells, nor is it likely to be simply a consequence of overexpression. Similar to data obtained with HEK293, ubiquitinated iNOS was detected in primary cells and in A549 cells without the need to block iNOS degradation with proteasomal inhibitors.

Murine iNOS Is Subject to Ubiquitination in RAW 264.7 Cells. We have previously demonstrated that, similar to human iNOS, murine iNOS is degraded primarily through the proteasome pathway (9). We investigated whether murine iNOS is also subject to ubiquitination before degradation. Cells from murine macrophage cell line RAW 264.7 were stimulated to produce iNOS by using lipopolysaccharide and IFN-γ (9). Coimmunoprecipitation of cell lysates with specific iNOS antibody, done on cell lysates, reveals the presence of iNOS-Ub conjugates (Fig. 4). This finding indicates that iNOS ubiquitination is a general mechanism of iNOS degradation for both murine and human isoforms.

Disruption of Ubiquitination, by a Dominant Negative Ubiquitin Mutant, Blocks iNOS Degradation. To investigate whether or not iNOS ubiquitination is required for its degradation, we used a construct that contains the ubiquitin mutant K48R. This mutant has the invariant Lys in position 48 mutated to Arg. The Lys 48 in ubiquitin is the site of isopeptide linkage of other ubiquitin molecules and is required for the completion of multiubiquitin chains that mark proteins for degradation (11, 17, 19). Expression of K48R ubiquitin mutant has a chain-terminating dominant negative effect, resulting in premature termination of ubiquitin chains. This action results in accumulation of "incompletely" ubiquitinated proteins that, although marked for degradation, are not properly targeted for degradation by proteasome (17, 19). HEK293T cells were cotransfected with plasmids containing cDNA of human iNOS and of either the ubiquitin mutant K48R or its empty vector. The molar ratio of iNOS plasmid to the coplasmid was 1:10, respectively. Cotransfection of K48R resulted in a time-dependent accumulation of iNOS (Fig. 5 A), suggesting that disruption of iNOS ubiquitination led to inhibition of its degradation. The ability of the K48R ubiquitin mutant to disrupt ubiquitination was independently verified by noting the accumulation of other ubiquitinated cellular proteins (Fig. 5*B*). In contrast, there was no change in levels of GAPDH (Fig. 5*C*), a protein that is known to be degraded by lysosomal pathway (30). These data suggest that ubiquitination of iNOS is required for its degradation.

Prevention of Ubiquitination in ts20 Cells Leads to iNOS Accumulation. ts20 is a mutant Chinese hamster lung cell line with a thermolabile ubiquitin-activating enzyme E1 (16–19). Cells are grown at the permissive temperature, 30°C. At the nonpermissive temperature, 40°C, E1 is irreversibly inactivated, leading to failure of ubiquitination to occur. E36 cells are the ts20 wild-type counterpart. We have generated ts20 and E36 cell lines that stably express human iNOS. To determine the consequence of failure of ubiquitination on iNOS degradation, we incubated cells for 24 h at 30°C or 40°C and analyzed cell lysates for expression of iNOS, E1, and GAPDH (Fig. 6*A*). Incubation of ts20 cells at 40°C resulted in concomitant accumulation of iNOS and marked reduction of E1. Similar changes were absent in wild-type E36 cells. In both cell lines, no changes were noted in GAPDH. These results suggest that a functional ubiquitin pathway is required for iNOS degradation.

Finally, we investigated the possibility that some nonubiquiti-

nated iNOS may still undergo proteasomal degradation. We reasoned that, in cells where ubiquitination is prevented, whether some nonubiquitinated iNOS is degraded by the proteasome, there would be a further increase in iNOS after proteasomal inhibition. We incubated ts20 and E36 cells in the absence or presence of the proteasome inhibitor MG132. At the permissive temperature 30°C, proteasomal inhibition led to iNOS accumulation in both ts20 and wild-type E36 cells (Fig. 6*B*), confirming that, in these cells, the proteasome pathway is the primary pathway for iNOS degradation. At the nonpermissive temperature 40°C, addition of MG132 to wild-type E36 cells led to marked increase in iNOS. In contrast, in ts20 cells in which iNOS degradation is already inhibited because of lack of ubiquitination, addition of MG132 did not lead to further increase in iNOS. These results suggest that disruption of ubiquitination of iNOS led to a ''maximal'' block of iNOS degradation similar to that seen with proteasomal inhibitors. They further confirm that ubiquitination is required for iNOS degradation by the proteasome.

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. Our results indicate that iNOS undergoes ubiquitination, which is required for its degradation. Potentially, 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). A recent study revealed that neuronal NOS is subject to ubiquitination (28). This finding may imply similar modes of degradation mechanisms between the various nitric oxide synthase isoforms. Future studies will address identification and characterization of the specific ubiquitin enzymes involved in the process of iNOS degradation. Our study lays the groundwork for such endeavors.

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