Accelerated ubiquitination and proteasome degradation of a genetic variant of inducible nitric oxide synthase

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Biochemical and pharmacological studies have suggested that NOS2 (inducible nitric oxide synthase) has a functional role in the blood pressure response to increases in dietary salt intake. On a high-salt diet, the Dahl/Rapp salt-sensitive (S) strain of rat, a genetic model of salt-sensitive hypertension, did not show increased nitric oxide production. NOS2 from S rats possesses a point mutation that results in substitution of proline for serine at position 714. In the present study, rat NOS2 was shown to be ubiquitinated *in vitro* and *in vivo* and to be degraded by the proteasome; this process was accelerated for the S714P mutant. Accelerated degradation of the S714P mutant enzyme accounted for the diminished enzyme activity of this mutant. Hsp90 (heat-shock protein 90) associated with NOS2 and modulated degradation, but was not responsible for the accentuated degradation of the S714P mutant enzyme. The combined findings demonstrate the integral role of ubiquitination and degradation by the proteasome in the regulation of NO production by rat NOS2. Demonstrating that this process is responsible for the abnormal function of the S714P mutant NOS2 in S rats confirms the physiological importance of the proteasome in NOS2 function.

Key words: Dahl/Rapp rat, geldanamycin, Hsp90, hypertension, nitric oxide, proteasome.

INTRODUCTION

The biological function and the mechanisms regulating the production of NO (nitric oxide) continue to be elucidated. NO is produced by three enzymes: a neuronal nitric oxide synthase (NOS1), an inducible nitric oxide synthase (NOS2), and an endothelial isoform of nitric oxide synthase (NOS3). Expression of NOS2, which has the capacity to produce large amounts of NO, is highly regulated by multiple events that occur during transcription [1–3], post-transcriptionally [4] and post-translationally [5–7]. The majority of the mechanisms that have been characterized involve synthesis and assembly of the active enzyme, and not degradation of NOS2.

The proteasome is a principal system involved in intracellular protein degradation. NOS1 is ubiquitinated and degraded by this system [8]. Addition of lactacystin, an inhibitor of proteasome degradation, to HEK293 cells transfected with human NOS2 and the RAW264.7 cell line prevented degradation of NOS2 [9]. The effect of lactacystin was independent of NO production by NOS2. The addition of inhibitors of other proteases, including calpain, trypsin and chymotrypsin, or lysosomal inhibitors did not alter NOS2 degradation. The physiological role of this process has not yet been explored.

The past three decades of research have shown that there is a genetic predisposition to the development of hypertension in response to a diet high in salt content. Perhaps Lewis Dahl and John Rapp have generated the most compelling data, by manipulating the genetic background of Sprague–Dawley (SD) rats to produce the Dahl/Rapp salt-sensitive (S) and salt-resistant (R) strains [10–13]. When placed on a high-salt diet, S rats consistently developed hypertension and end-organ damage, while R rats remained normotensive, despite the high salt intake [14]. Although the search for the genes that result in hypertension in S rats has not yet identified specific candidate genes, S rats have been shown to produce less NO in response to an increase in dietary salt [14,15]. Production of NO by NOS2 in the renal medulla [16] and by aortic smooth muscle cells (ASMC) in culture [17] was impaired in S rats. A point mutation in NOS2 has been identified, creating a serine-to-proline mutation at position 714. The mutation is unique to the S strain, and is responsible for diminished activity of NOS2 in S rats [17,18].

The purpose of the present study was to elucidate the functional role of the proteasome in NO production by NOS2 and to further identify the interaction of this process with the S714P mutation in NOS2. The findings afford a view of the physiological effect of ubiquitination and degradation of NOS2 by the proteasome.

Part of this work has been published in abstract form [18a].

MATERIALS AND METHODS

Preparation of rat ASMC and transfection of COS-7 cells

Primary cultures of ASMC were established by pooling thoracic aortas from four rats in each group (SD and S rats; obtained from Charles River Laboratories, Wilmington, MA, U.S.A.) using enzymic digestion techniques and culture conditions as described in [17]. Before use, cells in the 5th to 7th passage were incubated for 24 h in Dulbecco's minimal essential medium containing 10 % (v/v) fetal bovine serum with 30 μg/ml LPS (*Escherichia*

Abbreviations used: NOS1, neuronal nitric oxide synthase; NOS2, inducible nitric oxide synthase; NOS3, endothelial nitric oxide synthase; SD rat, Sprague-Dawley rat; S rat, Dahl/Rapp salt-sensitive rat; R rat, Dahl/Rapp salt-resistant rat; LPS, *Escherichia coli* lipopolysaccharide; LAC, *clasto*-lactacystin β-lactone; NO_x, nitrite plus nitrate; ASMC, aortic smooth muscle cells; Hsp90, heat-shock protein 90.

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coli lipopolysaccharide; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 150 ng/ml interferon-γ (Gibco BRL, Rockville, MD, U.S.A.).

Cultures of COS-7 cells (CRL-1651; American Type Culture Collection, Manassas, VA, U.S.A.) were simultaneously transfected using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA, U.S.A.) and 12 μg of pRc/CMV2 that contained the cDNA under study (wild-type NOS2 or the S714P or S714A mutant), as described previously [18]. Rat NOS2 cDNA was generously provided by Professor Göran K. Hansson (Karolinska Institute, Stockholm, Sweden). The S714P and S714A mutants were generated by site-directed mutagenesis and confirmed by direct sequencing [18]. Experiments were performed 48 h after transfection.

Determination of the metabolic fate of wild-type NOS2 and the S714P mutant

Following stimulation of ASMC from SD and S rats with LPS and interferon-γ, NOS2 was metabolically labelled by incubating the cells for 30 min in cysteine/methionine-free minimal essential medium (MEM Select-Amine Kit; Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 5 μM *clasto*-lactacystin β-lactone (LAC), the active metabolite of lactacystin [19–21], or 0.05 % DMSO (vehicle). Cells were then incubated in medium that contained 150 μCi/ml [^{35}S]cysteine/[^{35}S]methionine (Tran^{35}S-label; ICN Biochemicals, Irvine, CA, U.S.A.) for 2 h at 37 °C; medium also contained either LAC or vehicle. Labelling medium was then removed and the cells were washed with PBS and incubated at 37 °C in complete medium supplemented with LAC or vehicle. Cells were harvested at 0, 6, 12, 18 and 24 h. Aliquots of medium were also collected for quantification of NO_x (nitrite plus nitrate) production, which was determined as described previously using nitrate reductase and Griess reagent [22]. Cells were lysed in RIPA buffer containing protease inhibitors (10 μg/ml trypsin inhibitor, 100 μM leupeptin, 0.5 μM pepstatin A, 2 μg/ml aprotinin and 3 mM PMSF). The lysates were pre-cleared by incubating equal amounts of the samples with Protein A–Sepharose CL-4B beads (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) for 1 h at 4 °C. NOS2 was immunoprecipitated from the samples by incubation with anti-NOS2 monoclonal antibody (Transduction Laboratories) bound to Protein A–Sepharose CL-4B beads overnight at 4 °C. The precipitates were washed three times with RIPA buffer, and ^{35}S activity was quantified by liquid scintillation spectrometry. In other experiments, the precipitated beads were boiled in reduced Laemmli sample buffer, and resolved using SDS/PAGE (8 % polyacrylamide gels). The gels were dried and exposed to X-Omat film (Eastman Kodak Co.).

In vitro ubiquitination of NOS2

Wild-type NOS2 and the S714P and S714A mutants were purified from transiently transfected COS-7 cells using standardized procedures [23–26]. Lysates were obtained by resuspending the cells in ice-cold hypotonic buffer (20 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 8 mM KCl, 2 mM dithiothreitol, 0.5 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 10 μg/ml aprotinin) for 15 min. The cells underwent rapid freezing and thawing three times, followed by centrifugation at 14 000 ***g*** for 5 min at 4 °C. The resulting supernatant was ultracentrifuged at 100 000 ***g*** at 4 °C for 1 h. NOS2 was purified from these supernatant fractions by use of a 2′,5′-ADP–Sepharose 4B (Pharmacia Biotech) column, followed by a calmodulin affinity column. All manipulations were performed at 4 °C. The purity of the preparations was determined by SDS/PAGE, along with Western blotting to confirm the identity of the bands as NOS2. The concentration of protein in each sample was determined using a kit (Micro BCA Protein Assay; Pierce, Rockford, IL, U.S.A.).

The rate of conjugation of ^{125}I-ubiquitin to NOS2 was determined by incubating 5 μg of purified NOS2 for 2 h at 37 °C in 30 μl of ubiquitination mix. The protocol was similar to that published previously [27,28]. This reaction mixture contained 40 mM Tris/HCl, pH 7.6, 1 mM dithiothreitol, 10 % (v/v) glycerol, 0.15 pmol of ^{125}I-ubiquitin (9×10^5 c.p.m.), 1 μM ubiquitin aldehyde (100 ng/μl), 1 mg/ml methyl ubiquitin, 1 μM okadaic acid, an ATP-regenerating system (2 mM ATP, 5 mM $MgCl_2$, 10 mM phosphocreatine and 100 units/ml creatine kinase) and 1 mg/ml cytoplasmic protein extract from untransfected COS-7 cells. As described in [27], methyl ubiquitin was added because it competes with ubiquitin and terminates the polyubiquitination reaction of NOS2, thus permitting the appearance of discrete bands rather than a smear in the gels. At indicated times (0, 30, 60, 90 and 120 min), the reaction was halted by the addition of 200 μl of RIPA buffer. NOS2 was immunoprecipitated from each sample using identical conditions with 0.75 μg of mouse anti-NOS2 monoclonal antibody. The proteins were separated using SDS/6 %-PAGE and the gels were dried and exposed to X-Omat film (Eastman Kodak Co.). The bands were cut from the gel and the ^{125}I label was quantified using a gamma scintillation counter.

Co-immunoprecipitation analysis of interactions between NOS2, ubiquitin and Hsp90 (heat-shock protein 90)

Co-immunoprecipitation experiments were performed 48 h after transient transfection of COS-7 cells. Cell lysates (500 μl; 500 μg of total protein) were incubated at 4 °C for 2 h with 2 μg of anti-NOS2 monoclonal antibody (BD Transduction Laboratories, San Diego, CA, U.S.A.), 2 μg of anti-ubiquitin polyclonal antibody (Dako Corp., Carpinteria, CA, U.S.A.) or 2 μg of anti-Hsp90 monoclonal antibody (StressGen Biotechnologies Corp., Victoria, BC, Canada). Non-specific IgG (SouthernBiotech, Birmingham, AL, U.S.A.) was used as a control in the immunoprecipitation experiments. Samples were incubated overnight with 30 μl of Protein A–Sepharose CL-4B beads. The beads were washed three times with ice-cold RIPA buffer and then boiled in SDS sample buffer containing 6 mg/ml dithiothreitol. The proteins were resolved on SDS/6–8 %-PAGE and transferred to nitrocellulose membranes, which were probed with an anti-NOS2 monoclonal antibody, anti-ubiquitin polyclonal antibody, irrelevant IgG or anti-Hsp90 antibody. Prior to probing with the anti-ubiquitin antibody, the nitrocellulose membranes were autoclaved in distilled water for 20 min. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent and X-Omat film.

Determination of the effect of Hsp90 on NOS2 expression

At 48 h after transient transfection, COS-7 cells were exposed to 3 μM geldanamycin or 0.05 % DMSO (vehicle) for 24 h at 37 °C. Cell lysates were subjected to Western analysis using a mouse anti-NOS2 monoclonal antibody (Transduction Laboratories). Medium was harvested to determine NO_x production. In additional experiments, after incubation in geldanamycin or vehicle for 24 h, cells were metabolically labelled using techniques described above. Medium containing geldanamycin or vehicle was replaced and the experiment was terminated at 0, 6, 12, 18 and 24 h after metabolic labelling. Medium was harvested to determine

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NO_x production. NOS2 was immunoprecipitated from cell lysates using mouse anti-NOS2 monoclonal antibody bound to Protein A–Sepharose. The precipitates were washed three times with RIPA buffer and ^{35}S activity was quantified by liquid scintillation spectrometry. In other experiments, the precipitated beads were boiled in reduced Laemmli sample buffer and resolved using SDS/8 %-PAGE. The dried gels were exposed to X-Omat film and the density of each band was quantified using a densitometer.

Statistical analysis

Data are presented as means ± S.E.M. Statistical differences were determined using either Student's unpaired *t* test or one-way ANOVA with standard *post hoc* testing (Statview, version 5.0; SAS Institute, Inc., Cary, NC, U.S.A.) where appropriate. A P value of < 0.05 assigned statistical significance.

RESULTS

The half-life of S714P mutant NOS2 is decreased in ASMC in culture

Primary cultures of ASMC from S and SD rats were stimulated by incubation for 24 h in medium containing LPS and interferon-γ, which stimulates NOS2 expression in these cells [17]. Metabolic labelling of cytoplasmic proteins was then performed and the cells were incubated in LAC or vehicle overnight (Figure 1). The amount of immunoprecipitable ^{35}S-labelled NOS2 decreased more rapidly in vehicle-treated S cells containing the S714P mutant compared with vehicle-treated SD cells containing wild-type NOS2. In these experiments, the estimated half-life of the mutant protein in ASMC from S rats was lower than that of wild-type NOS2 present in SD cells (14.9 ± 2.0 compared with 28.5 ± 0.6 h; $P < 0.05$). Thus the biological half-life and functional activity of NOS2 in cells from S rats, which contained the S714P mutant [17,18], was reduced compared with that of wild-type NOS2 in cells from SD rats. This difference was abolished following addition of LAC to the medium. NO_x production by ASMC from S rats was diminished compared with that by ASMC from SD rats, but the deficit was corrected by addition of LAC (Figure 1).

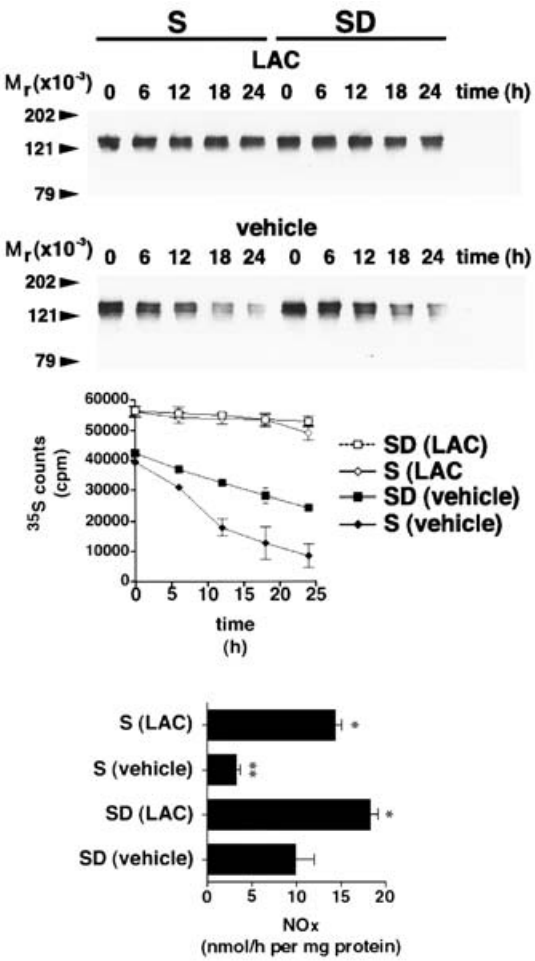

Figure 1 Metabolic labelling study of NOS2 in ASMC from S and SD rats

Cells were exposed to either 5 μM LAC or 0.05 % DMSO (vehicle) 30 min before metabolic labelling. The autoradiogram from a single experiment (top) shows persistent expression of ^{35}S-labelled NOS2 following LAC treatment, compared with the vehicle-treated cells. Positions of molecular mass markers (kDa) are shown on the left. The middle panel shows counts obtained from these studies ($n = 3$ experiments in each group). Treatment with LAC resulted in persistent and similar expression of ^{35}S-labelled NOS2 in both S and SD cells over the course of the experiment. In contrast, while the values did not differ at the initiation of the experiment, by 6 h of observation, removal of ^{35}S-labelled NOS2 was increased ($P < 0.05$) in ASMC from S rats compared with ^{35}S-labelled NOS2 in ASMC from SD rats. The bottom panel demonstrates that vehicle-treated ASMC from S rats produced less ($P < 0.05$) NO_x than vehicle-treated ASMC from SD rats. Addition of LAC increased NO_x production. The increase in NO_x was demonstrated because NO_x was determined by harvesting the medium at the end of the experiment; the result therefore was an integration of NOS2 function over the 24 h of the experiment. Significance: $*P < 0.05$ compared with vehicle-treated cells; ‡$P < 0.05$ compared with vehicle-treated ASMC from SD cells.

Rat NOS2 is polyubiquitinated, and *in vitro* ubiquitination of S714P NOS2 is accentuated compared with that of wild-type and S714A NOS2

Co-immunoprecipitation studies using transiently transfected COS-7 cells were used to determine ubiquitination of wild-type NOS2, S714P NOS2 (found in S rats [17]) and S714A NOS2 (Figure 2). These experiments used commercially available antibodies directed against ubiquitin and NOS2. Immunoprecipitation of NOS2 using anti-NOS2 antibody followed by Western analysis using anti-ubiquitin antibody demonstrated the presence of ubiquitin on NOS2. Similar findings were observed when the anti-ubiquitin antibody was used to immunoprecipitate the cytoplasmic proteins followed by Western analysis using anti-NOS2. The high-molecular-mass bands confirmed the presence of multiple ubiquitin molecules bound to NOS2, consistent with polyubiquitination.

Using standard techniques, wild-type and mutant forms of NOS2 were purified from COS-7 cells expressing these proteins and used to quantify rates of ubiquitination during incubation *in vitro* with ^{125}I-ubiquitin (Figure 3). An increase in the rate of ubiquitination of the S714P mutant was apparent ($P < 0.05$) by the 30 min time point. The rate of ubiquitination of the S714P mutant NOS2 was 139.3 ± 13.5 % of that of wild-type NOS2, while the ubiquitination rate of the S714A mutant was not altered (100.9 ± 1.7 % of wild-type NOS2).

Hsp90 interacts with NOS2 and inhibits degradation

Because previous studies have shown that Hsp90 is directly involved in promoting degradation by the proteasome of mutant insulin receptors [29], co-immunoprecipitation experiments were performed initially to demonstrate association of Hsp90 with

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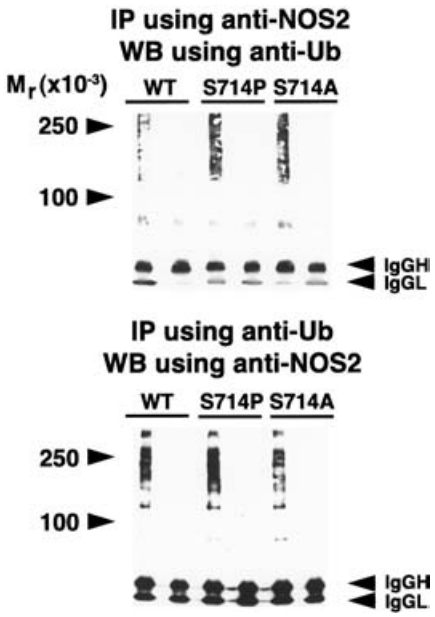

Figure 2 Co-immunoprecipitation experiments using cytoplasmic extracts of transiently transfected COS-7 cells

LAC (5 μM) was added to the medium 2 h prior to harvesting the lysates. The upper panel shows the results obtained using a monoclonal anti-NOS2 antibody to immunoprecipitate (IP) proteins from the cytoplasm, followed by Western blot (WB) analysis using a polyclonal anti-ubiquitin (Ub) antibody. The lower panel shows the results using anti-Ub to immunoprecipitate the proteins, followed by Western blot using anti-NOS2. Specific antibodies were used in lanes 1, 3 and 5, while an irrelevant IgG was used in lanes 2, 4 and 6. Both gels demonstrate that ubiquitin interacts with all three NOS2 proteins, i.e. wild type (WT), and S714P and S714 A mutants. Positions of molecular mass markers (kDa) are shown on the left. IgGH, immunoglobulin heavy chain; IgGL, immunoglobulin light chain.

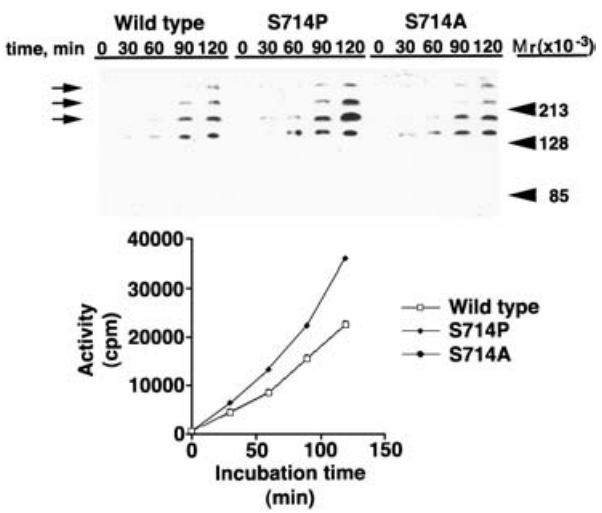

Figure 3 *In vitro* ubiquitination reaction using ^{125}I-ubiquitin and purified NOS2 as the substrate

NOS2 was immunoprecipitated after the incubation, followed by electrophoresis using SDS/PAGE. The amount of NOS2 and the conditions for immunoprecipitation were identical for all samples. The autoradiogram (upper panel) demonstrates polyubiquitination (arrows) of all three proteins; however, compared with wild-type NOS2 and the S714A mutant, the amount of ^{125}I-ubiquitin incorporated on to the S714P mutant was increased ($P < 0.05$) by the 30 min time point and remained elevated throughout the study. The lower panel shows ^{125}I-ubiquitin incorporation (mean $\pm$ S.E.M.) at each time point ($n =$ five experiments in each group). The curves representing incorporation of ^{125}I-ubiquitin on to wild-type NOS2 and the S714A mutant overlapped and did not differ at any time point following initiation of the reaction.

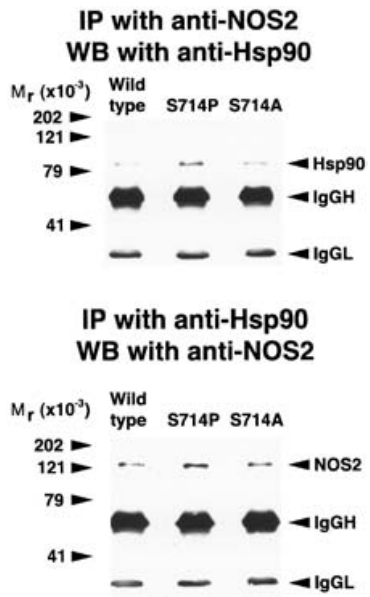

Figure 4 Co-immunoprecipitation experiments using cytoplasmic extracts of transiently transfected COS-7 cells

The upper panel shows results obtained using a monoclonal anti-NOS2 antibody to immunoprecipitate (IP) proteins from the cytoplasm, followed by Western blot (WB) analysis using a monoclonal anti-Hsp90 antibody. The lower panel shows results obtained using anti-Hsp90 to immunoprecipitate the proteins, followed by Western blot using anti-NOS2. Both gels demonstrate that Hsp90 interacted with all three NOS2 proteins. Positions of molecular mass markers (kDa) are shown on the left. IgGH, immunoglobulin heavy chain; IgGL, immunoglobulin light chain.

NOS2 in the cytoplasm (Figure 4). Wild-type NOS2 and the S714P and S714A mutants bound Hsp90. Addition of geldanamycin, an inhibitor of Hsp90, decreased expression of wild-type NOS2 and the S714A mutant, and further decreased expression of the S714P mutant (Figure 5). NO_x production rates correlated with changes in NOS2 expression (Figure 5). Metabolic labelling experiments were then performed in transiently transfected COS-7 cells exposed to geldanamycin or vehicle (Figure 6). Addition of geldanamycin decreased the protein half-life of both wild-type NOS2 and the S714P mutant.

DISCUSSION

Regulation of the eukaryotic proteome is a dynamic process that balances synthesis and degradation. A large number of cytoplasmic proteins are degraded by the proteasome. This process is usually initiated by ubiquitination, i.e. the conjugation of ubiquitin to proteins. Ubiquitination targets a protein for ubiquitin-mediated degradation by the proteasome [30–33]. Targeting proteins for ubiquitination is complicated and incompletely understood. Because NOS2 is a cytoplasmic protein, it may be subject to ubiquitination. Using inhibitors of cellular degradation pathways, Musial and Eissa [9] demonstrated that human and murine NOS2 were degraded by the proteasome. The present study confirmed these findings in the rat, as well as our previous studies that used a heterologous system [18], and showed that rat NOS2 is polyubiquitinated *in vitro* and *in vivo* and degraded by the proteasome. Since an S714A mutant NOS2 was ubiquitinated and degraded in a fashion similar to wild-type NOS2, the process does not

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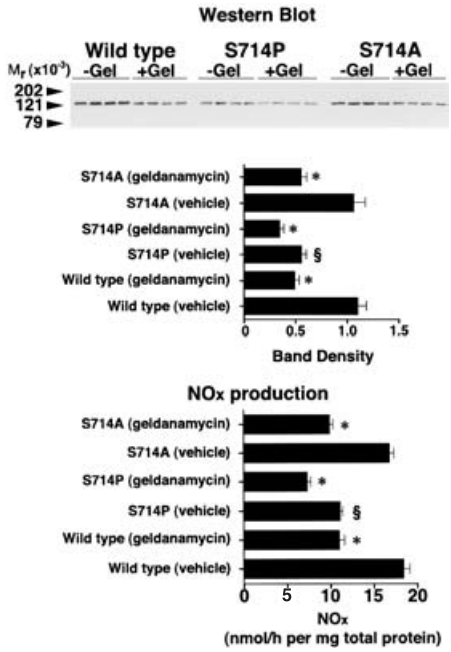

Figure 5 Western analysis (top panel) of cytoplasmic extracts from transiently transfected COS-7 cells expressing wild-type NOS2 and the S714P and S714A mutants

The cells were exposed to 3 μM geldanamycin (+Gel) or 0.05 % DMSO (−Gel) for 24 h before study. The results were quantified (middle panel; $n = 4$ in each group). Addition of geldanamycin decreased expression of all three NOS2 proteins. The bottom panel shows NO_x production by these cells. As expected, NO_x production was impaired in vehicle-treated COS-7 cells expressing the S714P mutant. Addition of geldanamycin decreased NO_x production by all three groups. Significance: $^*P < 0.05$ compared with corresponding vehicle-treated cells; §$P < 0.05$ compared with the other two vehicle-treated groups.

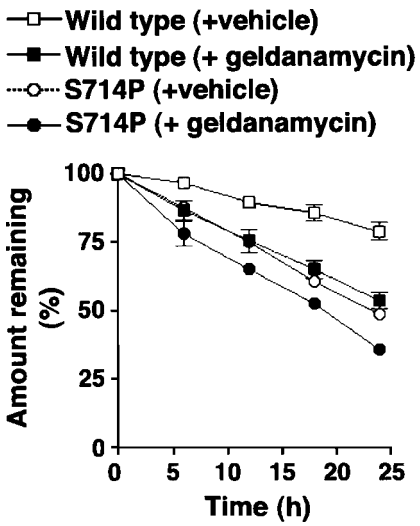

Figure 6 Metabolic labelling studies using COS-7 cells transiently transfected with wild-type NOS2 or the S714P or S714A mutant

In these experiments, after incubation in geldanamycin or vehicle for 24 h, cells were metabolically labelled and again placed in medium containing geldanamycin or vehicle. The experiment was terminated at 0, 6, 12, 18 and 24 h after metabolic labelling and the results normalized to the initial value ($n = 3$ experiments in each group). Addition of geldanamycin accelerated the decline in both wild-type NOS2 and the S714P mutant. By 24 h, the amount of wild-type NOS2 in geldanamycin-treated cells had declined to 53.6 ± 3.0 % of baseline, compared with 78.5 ± 3.1 % in vehicle-treated cells expressing wild-type NOS2 ($P < 0.001$). The decrease from baseline for the S714P mutant was also greater in geldanamycin-treated cells than in vehicle-treated cells (35.4 ± 1.7 % and 48.6 ± 1.4 % remaining respectively; $P < 0.005$).

appear to involve phosphorylation of this residue. Novel findings in the present paper include the demonstration that ubiquitination of S714P mutant NOS2, which is present in S rats [17], was accelerated *in vitro*. Another new finding is the demonstration that the degradation process was accentuated for the S714P mutant present in ASMC from S rats, and was responsible for the diminished enzyme activity of this mutant. Despite the multiple levels of control of NOS2 activity [1–7], it is perhaps surprising that the proteasome is involved in determining NOS2 activity *in vivo*. However, inhibition of the proteasome promoted increases in NO_x production by vascular smooth muscle cells from both S and SD rats, thus supporting a function of the proteasome in NOS2 activity in vascular smooth muscle cells.

The role of the molecular chaperone, Hsp90, in NOS2 degradation was also examined. Imamura and associates [29] reported that Hsp90 bound mutant insulin receptors and was directly responsible for their accentuated degradation in the proteasome. Another heat-shock protein, Hsc70 (heat-shock cognate 70 stress protein), facilitates the ubiquitination and degradation of a variety of cellular proteins [34]. However, Hsp90 has been shown to bind and facilitate the activity of NOS3, the endothelial isoform of NOS [35–37]. Binding of Hsp90 to NOS1 decreased turnover of this enzyme [38]. The present study demonstrates that wild-type and two mutant forms of NOS2 associated directly with Hsp90. These immunoprecipitation experiments were supported by experiments showing that geldanamycin, which disrupts the action of Hsp90 [39], further decreased the activity and half-life of both wild-type NOS2 and the S714P mutant. Thus Hsp90 bound both wild-type NOS2 and the S714P mutant, but did not facilitate degradation; instead it functioned to increase the protein half-life of NOS2.

In summary, together with previous studies showing that the affinity of mutant NOS2 for L-arginine does not differ from that of the wild-type enzyme [18], the present results demonstrate that accelerated degradation by the proteasome is responsible for the shortened half-life and diminished function of the S714P mutant NOS2 observed in S rats. In addition, because LAC completely repaired the defect in NO production by this mutant, the mutation does not affect other events that might regulate function, such as phosphorylation of NOS2. The present findings therefore demonstrate the potential importance of the proteasome in the regulation of NO production by NOS2. Using inhibitors of NOS2, Tan and associates [40] and Rudd et al. [41] independently verified a role for NOS2 in the development of salt-sensitive hypertension. While the present findings do not demonstrate the pathological importance of the defect in NO production in salt-sensitive hypertension in S rats, the present data add to existing studies showing a defect in NO production by the S714P mutant, which is present in S rats [17,18], supported by a recent analysis suggesting that the *Nos2* gene resides within a strong quantitative trait locus on chromosome 10 of S rats [42]. It is also possible that this mutation plays a significant role in other pathology, such as the chronic kidney disease that is prevalent in this rat strain [14]. The precise role of this genetic variation of NOS2 in salt-sensitive hypertension in the Dahl/Rapp rat awaits confirmation. Finally, because salt-sensitive hypertension in this model is not a monogenic disorder, the present data do not exclude other functional genetic abnormalities in these rats.

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