S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities

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Edited by Louis J. Ignarro, University of California School of Medicine, Los Angeles, CA, and approved April 27, 2005 (received for review October 1, 2004)

Nitric oxide is implicated in a variety of signaling pathways in different systems, notably in endothelial cells. Some of its effects can be exerted through covalent modifications of proteins and, among these modifications, increasing attention is being paid to S-nitrosylation as a signaling mechanism. In this work, we show by a variety of methods (ozone chemiluminescence, biotin switch, and mass spectrometry) that the molecular chaperone Hsp90 is a target of S-nitrosylation and identify a susceptible cysteine residue in the region of the C-terminal domain that interacts with endothelial nitric oxide synthase (eNOS). We also show that the modification occurs in endothelial cells when they are treated with *S*-nitroso-L-cysteine and when they are exposed to eNOS activators. Hsp90 ATPase activity and its positive effect on eNOS activity are both inhibited by S-nitrosylation. Together, these data suggest that S-nitrosylation may functionally regulate the general activities of Hsp90 and provide a feedback mechanism for limiting eNOS activation.

atherosclerosis | nitrosation | vascular wall | chaperone

Recent years have witnessed an increasing interest in the roles of nitric oxide (NO) in signal transduction pathways other than its activation of the cGMP pathway. Many of these roles rely on NO's ability to alter protein function through posttranslational modifications. Among these modifications, S-nitrosylation has emerged as a potential and fundamental regulator of protein function. S-nitrosylation is a covalent modification of thiol groups by formation of a thionitrite (-S-N=O) group, facilitated by the formation of higher nitrogen oxides (1, 2). To date, several dozens of proteins have been shown to become S-nitrosylated and, in many cases, this modification was accompanied by altered function (see table S1 of ref. 1 for review).

Nitric oxide, synthesized in the endothelium by endothelial nitric oxide synthase (eNOS), plays crucial roles in the vascular wall, including the maintenance of vascular tone. The possibility that NO might modify eNOS, or elements of the complex system involved in its activation, is an attractive hypothesis, suggesting a potential autoregulatory feedback mechanism. The eNOS enzyme is regulated by several posttranslational modifications including myristoylation, palmitoylation, and phosphorylation (3). This enzyme is also tightly regulated by specific interactions with inhibitory proteins such as caveolin-1 and by positive modulation by the scaffolding protein Hsp90. These interactions have been described in detail, and a relatively complete picture is beginning to emerge (4).

We have previously used a proteomic approach to identify several proteins that were S-nitrosylated after exposure of vascular endothelial cells to the physiological nitrosothiol, *S*-nitroso-L-cysteine (CSNO) (5). Further work led to the identification of Hsp90 as a protein susceptible to S-nitrosylation. This chaperone protein, known for its functions in protein folding, degradation, and scaffolding, has attracted renewed interest for its role in signal transduction (6). Very recently it has also been implicated in the regulation of tumor growth (7).

Here we show the S-nitrosylation of Hsp90, both of the purified protein and in endothelial cells. We have identified a specific thiol residue involved in this modification, and we have also studied its functional consequences. S-nitrosylation of Hsp90 abolishes the positive regulation on eNOS activity mediated by native Hsp90. Furthermore, S-nitrosylation of Hsp90 also has critical consequences for other intrinsic properties of this chaperone, such as its ability to hydrolyze ATP.

Materials and Methods

***In Vitro* Detection of S-Nitrosylation in Hsp90 by Ozone Chemiluminescence.** Recombinant purified Hsp90α was allowed to react in the presence of $NaNO_2$ in 0.5 M HCl (molar ratio Hsp90:NO_2^- 1:2) for 20 min at room temperature. The reaction was stopped (and the nitrosylating agent eliminated) by shifting to pH 7.4 with 5.0 M NaOH and PBS. Alternatively, the protein was incubated in PBS with 1 mM *S*-nitrosoglutathione (GSNO) or 1 mM diethylamine-NONOate for 20 min at room temperature.

S-Nitrosylation was determined by chemical reduction and chemiluminescence as described in ref. 8. GSNO-treated protein was first separated from GSNO by passing the sample through a Sephadex G-25 column. The sample was injected into an 8-ml anaerobic solution containing 100 μM CuCl, 1 mM cysteine (pH 6) at 50°C, and purged continuously with nitrogen in a Sievers 280 Nitric Oxide Analyzer. Stability of the modification was studied by analysis of the time course of the signal, obtaining a half-life for the S-nitrosylated protein of ≈120 min at 37°C.

Biotin Switch Assay. Determination of S-nitrosylation by the biotin switch assay was performed as described in refs. 5 and 9, either on *in vitro* treated protein after precipitation with acetone or on treated cells extracts. Details are given in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Analysis of S-Nitrosylation by Mass Spectrometry. Five micrograms of recombinant human Hsp90α were S-nitrosylated with 1 mM GSNO or 110 pmol NO_2^- as above, subjected to cold acetone precipitation, lyophilized to dryness, and dissolved in 20 μl of

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CSNO, *S*-nitroso-L-cysteine; eNOS, endothelial nitric oxide synthase; GSNO, *S*-nitrosoglutathione.

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denaturing solution (8 M urea/25 mM ammonium bicarbonate, pH 8.0). The mixture was diluted 5-fold to reduce urea concentration and digested with trypsin (1:50 protease to protein ratio) at 37°C for 4 h. The tryptic peptide pool was lyophilized to dryness, dissolved in solvent A (0.5% acetic acid), and desalted by consecutive runs on two 1 mm × 5 mm C18 MicroPrecolumns (LC Packings, Amsterdam) at a flow of 5 μl/min on a Smart microHPLC system (Amersham Pharmacia, Uppsala) equipped with a flow splitter and working at 60 μl/min. Peptides were eluted in a single step with 95% solvent B (0.5% acetic acid/80% acetonitrile). The tryptic peptide pool was then lyophilized to dryness to remove the acetonitrile, dissolved in 0.5% acetic acid, and analyzed by RP-HPLC-MS/MS on a Surveyor HPLC system and a 0.18 mm × 150 mm BioBasic 18 RP column (Thermo-Keystone, Bellefonte, PA), operating at ≈1.5 μl/min, connected online with a LTQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA). Peptides were eluted by using a 90-min gradient from 5% to 60% solvent B and selectively monitored by continuous MS/MS scans over the masses corresponding to the non-S-nitrosylated peptide (1,161.5 Da), the S-nitrosylated peptide (1,181.7 Da), and another Hsp90-derived peptide used as an internal control (917.4 Da). The isolation width was 3 atomic mass unit, and the normalized collision energy was 35%.

Structural Modeling. Human Hsp90α C-terminal domain structure model was built with the SWISS-MODEL protein homology-modeling server (10) (http://swissmodel.expasy.org) by using as a template the eight chains of the PDB ID code 1sf8, corresponding to the structure of the *Escherichia coli* HtpG C-terminal domain (11). Ribbon representation was obtained with the MOLMOL program (12).

Detection of S-Nitrosylated Hsp90 in Endothelial Cells by Biotin Derivatization or Immunoprecipitation Coupled to Immunoblotting. Extracts from endothelial cells were adjusted to 0.5 mg/ml protein and subjected to either immunoprecipitation with an anti-S-nitrosocysteine polyclonal antibody or to the biotin switch assay and purification of biotinylated proteins as described in refs. 5 and 9. In both cases, Hsp90 was identified by Western blot by using the specific antibody.

ATPase Assay. ATPase activity was assayed at 37°C by using pyruvate kinase and lactate dehydrogenase to couple ADP production to the oxidation of NADH and monitored as a decrease in absorbance at 340 nm (13). The reaction mixture was 200 mM Tris (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 120 μM NADH, 12.5 units of pyruvate kinase, 10.45 units of lactate dehydrogenase, and 1 mM ATP. To start the reaction, Hsp90 was added (1 μM). Absorbance decrease was recorded at 340 nm in an Ultrospec 4000 spectrophotometer (Amersham Pharmacia Biotech, Uppsala). Specific ATPase activity was 0.47 ± 0.07 min^{-1}. This value is in the range obtained for yeast Hsp90 ATPase activity with the same assay (14). Geldanamycin (2 μM) was used as control, because it is a specific inhibitor of Hsp90.

NOS Activity Assay. The conversion of ^{3}H-labeled L-arginine to ^{3}H-labeled L-citrulline was used to determine the activity of purified recombinant eNOS. Hsp90 was S-nitrosylated with $NaNO_2$ as previously described, pH was neutralized, and S-nitrosylation was assessed by ozone chemiluminiscence, giving a *S*-nitrosothiol content of ≈1 mol per mol of Hsp90. Control Hsp90 was treated in parallel with acid in the absence of $NaNO_2$. After incubation of eNOS (1 μM) with Hsp90 or S-nitrosylated Hsp90 (1 μM) for 10 min at room temperature, eNOS activity was determined by measuring the radioactivity that passed through a cation exchange column, corresponding to the labeled L-citrulline produced (15). The reaction buffer was Tris·HCl 50 mM (pH 7.4) containing 5 μM FAD, 5 μM FMN, 1 mM NADPH, 2.5 mM $CaCl_2$, 10 μg/ml calmodulin, and 0.1 μCi (1 Ci = 37 GBq) ^{3}H-L-Arg.

Supporting Information. Chemicals, cell culture, *in vivo* treatment, and protein extraction are described in *Supporting Materials and Methods*.

Results

Detection of *in Vitro* S-Nitrosylation. Earlier proteomic experiments, based on the biotin switch assay (5), showed that Hsp90α is S-nitrosylated in endothelial cells, both when cell extracts are treated with GSNO and when intact cells are treated with CSNO (Fig. 6, which is published as supporting information on the PNAS web site). While this paper was under review, there have been other reports on the proteomic identification of Hsp90 S-nitrosylation in macrophages treated with nitrosothiols (16, 17) and in *Mycobacterium tuberculosis* (htpG, homolog of Hsp90) (18).

To confirm this modification, we treated purified Hsp90α with acidified NO_2^-, the NO donor diethylamine-NONOate, or a nitrosothiol (GSNO). A clear band was detected when it was subjected to the biotin switch assay (Fig. 1*A*).

Treatment with Cu^+/cysteine at pH 6 allows specific reduction of *S*-nitrosothiols to liberate NO, which can be measured on a nitric oxide analyzer. We performed this assay on recombinant Hsp90α treated with either GSNO (Fig. 1*B*) or acidified NO_2^- (Fig. 1*C*). In both cases, NO liberation was detected, indicating that these treatments S-nitrosylated the protein. In the case of GSNO, as this nitrosothiol can also be detected with this methodology, the sample was separated by gel filtration, allowing us to distinguish peaks corresponding to the S-nitrosylated proteins from the low molecular mass fractions containing GSNO (Fig. 1*B*).

Identification of S-Nitrosylated Cysteine Residue(s) of Hsp90 by Mass Spectrometry. To identify the residues of Hsp90 that are modified by S-nitrosylation, the purified protein was subjected to *in vitro* nitrosylation with acidified NO_2^- or with GSNO as above and digested with trypsin in solution. The tryptic peptide pool was then analyzed by liquid chromatography on line with electrospray ionization and detection by linear ion trap mass spectrometry. Peptide ions were identified in survey scans and automatically subjected to collision-induced fragmentation. The fragment MS/MS spectra containing potentially significant sequence information were analyzed for the presence of S-nitrosylated Cys residues. In both treatments, a single peptide with 1,181.7 Da was observed to produce a MS/MS spectra that appeared to be consistent with the expected modification (data not shown). To obtain more conclusive evidence, these analyses were repeated by performing continuous ion fragmentation analysis on this peptide ion, taking advantage of the scanning speed of the linear ion trap, to increase the signal-to-noise ratio by a more intensive spectrum averaging. As shown in Fig. 2*A*, the fragment spectra clearly demonstrated the presence of nitrosylation in one of the two contiguous cysteines of the peptide, Cys 596 and Cys 597. Based on the presence of the b_6^+ ion (Fig. 2*A*), nitrosylation can be putatively assigned to Cys 597, although this ion was always generated with a low yield, so nitrosylation at Cys 596 could not be definitively ruled out in these experiments.

Interestingly, these cysteine residues are in the region of Hsp90 that has been described to interact with eNOS (Fig. 2*B*; ref. 19). They have also been previously identified as reactive cysteines in rat Hsp90 (20). We have built a model of the Hsp90α region spanning the mapped cysteines (Fig. 2*C*) based on the recently published structure of its *E. coli* homolog, htpG (11). In this model, the two contiguous cysteines are arranged in a β-chain, with the Cys 596 side chain oriented toward the domain

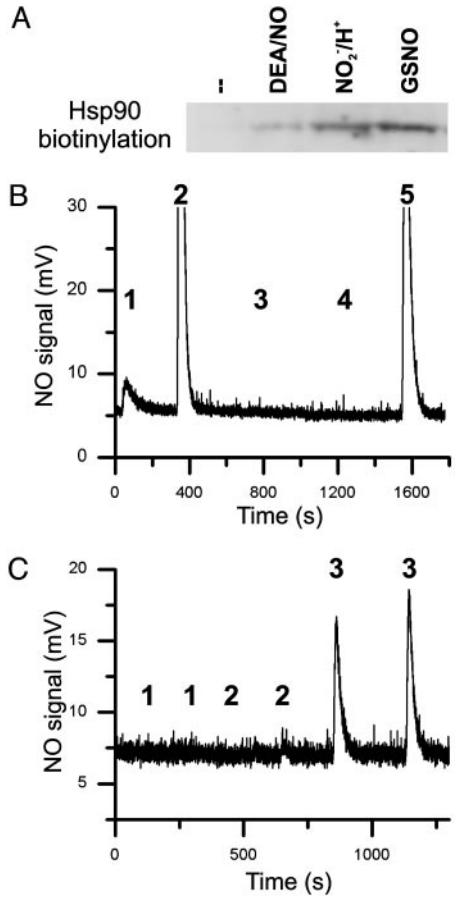

Fig. 1. Detection of S-nitrosylation by the biotin switch technique and by specific reduction and ozone chemiluminescence. (*A*) Recombinant human Hsp90α (1.3 μM) was left untreated (−) or treated with 1 mM diethylamine-NONOate (DEA/NO), 2.7 μM $NaNO_2$ in 0.5 M HCl (NO_2^-/H^+), or 1 mM GSNO. Hsp90α preparations were then subjected to the biotin switch assay, and biotinylated protein was detected by Western blot with streptavidin. The image shown is representative of three independent experiments. (*B* and *C*) Recombinant human Hsp90α was reduced with Cu^+/cysteine, and NO release detected in a nitric oxide analyzer apparatus. Figures shown are representative of at least three independent experiments. (*B*) Protein (5.3 μM) or buffer was treated with 1 mM GSNO, and protein and low molecular-mass (LMM) fractions were separated in a Sephadex G-25 column before analysis. 1: Hsp90 plus GSNO, protein fraction; 2: Hsp90 plus GSNO, LMM fraction; 3: Hsp90, protein fraction; 4: buffer plus GSNO, protein fraction; 5: buffer plus GSNO, LMM fraction. (*C*) Protein (0.8 μM) was treated with 1.6 μM $NaNO_2$ in 0.5 M HCl or HCl only for 20 min and adjusted to pH 7.4 with NaOH and PBS before analysis. 1: Buffer plus $NaNO_2$; 2: protein without $NaNO_2$; 3: protein plus $NaNO_2$.

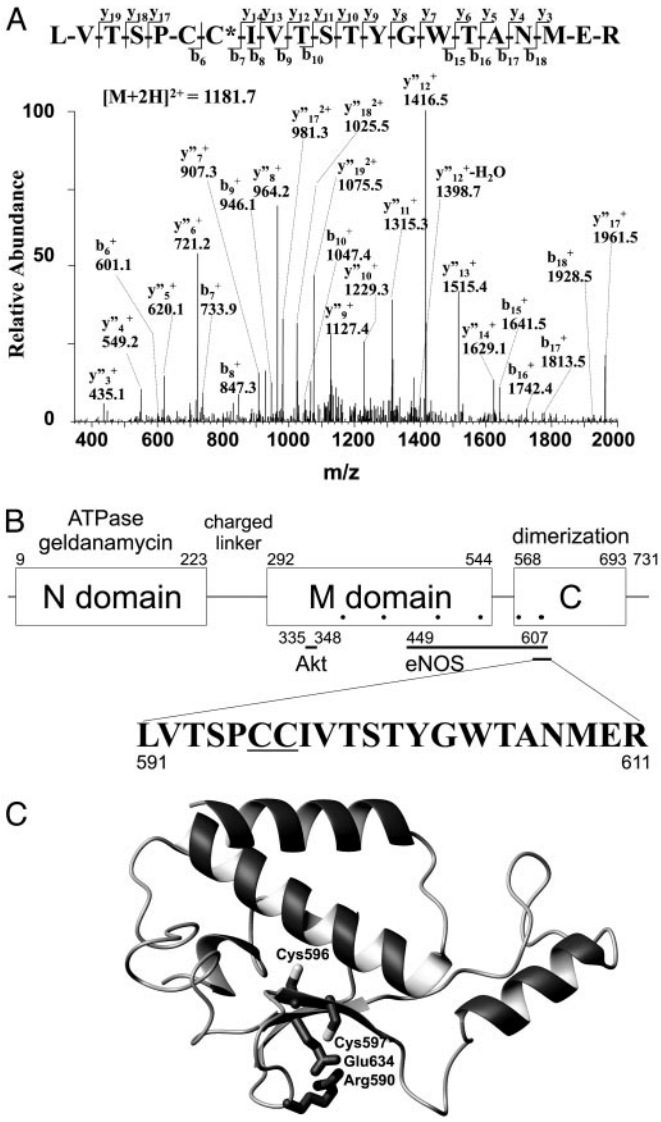

Fig. 2. Detection and identification of S-nitrosylation by mass spectrometry. (*A*) Recombinant human Hsp90α was either treated with $NaNO_2$ (1:2 molar ratio) in 0.5 M HCl and adjusted to pH 7.4 with NaOH and PBS or with 1 mM GSNO in PBS. Samples were then trypsin digested and analyzed by LC-MS by using a linear ion trap detector, as described in *Materials and Methods*. Shown is the fragmentation spectrum of the S-nitrosylated peptide corresponding to residues 591–611 of Hsp90α, together with the assignation of the fragmentation series to the sequence of the modified peptide. *, tentative S-nitrosylated Cys. (*B*) Schematic of Hsp90α domains (in boxes) and the localization of the cysteine residues (dots) and the S-nitrosylated peptide detected by MS. Functions assigned to the domains are written above, and regions described to interact with Akt and eNOS are shown below. Numbers correspond to the human Hsp90α sequence and, in the case of the domains, they denote the boundaries of the crystallographic structures. (*C*) Ribbon representation of the C-domain structure model of human Hsp90α (residues 568 to 693 of SWISS-PROT entry P07900), based on the structure of the C-terminal domain of *E. coli* htpG (PDB ID code 1fs8D). The side chains of cysteines 596 and 597, arginine 590, and glutamic acid 634 are highlighted.

inside, whereas Cys 597 is clearly exposed to the protein surface. Also, the Cys 597 thiol is close to the Arg 590 and Glu 634 side chains, which could provide an environment favoring its S-nitrosylation by an acid-base mechanism. This prediction also supported the notion that S-nitrosylation most probably takes place at the Cys 597 residue.

To discriminate between these two residues, a mutant of Cys 597 to serine was constructed, produced in *E. coli*, and partially purified. After treatment with 1 mM GSNO, the nitrosylated form of the peptide was found by MS only for the wild-type protein (Fig. 7, which is published as supporting information on the PNAS web site), ruling out the possibility that Cys 596 were S-nitrosylated.

A biotin switch performed on partially purified proteins revealed a distinct behavior of the C597S mutant; the untreated protein gives a basal signal, whereas there is no increase after GSNO treatment (data not shown). When S-nitrosylation of these proteins was analyzed by ozone chemiluminescence, none of the untreated proteins gave any nitrosothiol signal. After treatment with GSNO and separation by gel filtration, *S*-nitrosothiol signal was clearly reduced in the case of the C597S mutant compared with the signal of the wild-type (Fig. 8, which

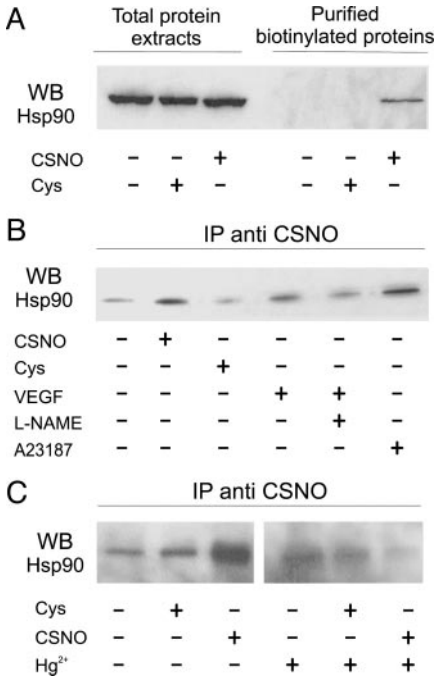

Fig. 3. Detection of S-nitrosylated Hsp90 in endothelial cells. (*A*) EA.hy926 cells were untreated (−) or exposed (+) to 1 mM cysteine (Cys) or S-nitrosocysteine (CSNO) for 15 min. Protein extracts were subjected to the biotin switch technique, and the biotinylated proteins were purified with avidin-agarose, followed by elution with 2-mercaptoethanol. Hsp90 was detected in total protein extracts and in eluates by SDS/PAGE and immunoblot. (*B*) EA.hy926 cells were untreated, treated with Cys or CSNO (1 mM), or stimulated with VEGF (50 ng/ml) with or without prior incubation with L-NAME (0.5 mM, 1 h) or calcium ionophore A23187 (10 mM) for 15 min. Protein extracts were immunoprecipitated with anti S-nitrosocysteine and Hsp90 identified by immunoblot. (*C*) Extracts from EA.hy926 cells untreated or exposed to Cys or CSNO (1 mM) were immunoprecipitated with anti-S-nitrosocysteine with or without prior specific breakdown of the S-NO bond by Hg^{2+} and Hsp90 identified by immunoblot. Figures shown are representative of at least two independent experiments.

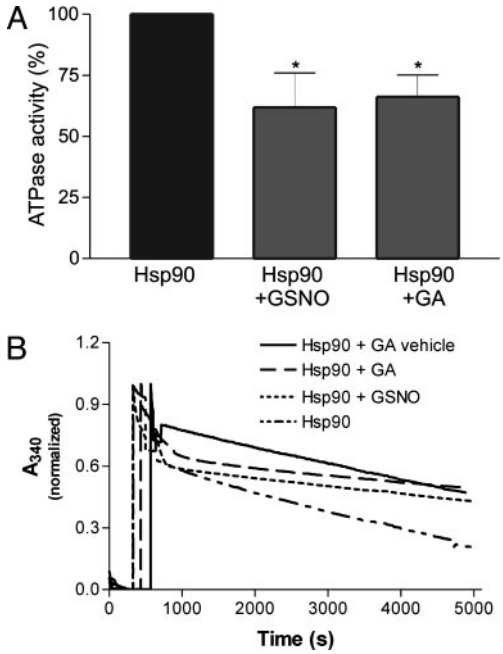

Fig. 4. Hsp90 ATPase activity is inhibited by S-nitrosylation. Hsp90 was 1 μM throughout. (*A*) Activities are shown for Hsp90 alone, after S-nitrosylation with 1 mM GSNO ($n = 7$), or incubation with the Hsp90-specific inhibitor, geldanamycin (GA; 2 μM, $n = 4$). ATPase activity is expressed as a relative slope to that of Hsp90 alone. Values are means ± SEM; *, $P < 0.05$ with respect to Hsp90 alone. (*B*) A representative experiment showing experimental progress curves corresponding to NADH consumption after addition of Hsp90 alone (in Tris buffer), Hsp90 plus a GA vehicle (DMSO), Hsp90 preincubated with GSNO, or Hsp90 plus GA.

is published as supporting information on the PNAS web site). These results reveal that Cys 597 is a clear target for S-nitrosylation.

Detection of S-Nitrosylated Hsp90 in Cells. To detect the presence of S-nitrosylated Hsp90 *in vivo*, endothelial cells were initially treated with CSNO, subjected to the biotin switch assay, and identified by immunoblot. As shown in Fig. 3*A*, after the biotin purification step, a clear band could be detected in extracts from CSNO-treated cells.

In a complementary approach, extracts of treated cells were immunoprecipitated with an antibody capable of detecting S-nitrosylated thiols (21, 22), and Hsp90 was detected by immunoblot (Fig. 3*B*). A clear increment is seen with CSNO treatment and eNOS activators (VEGF and the calcium ionophore A23187), whereas pretreatment with the NOS inhibitor L-NAME abolished the effect of VEGF (Fig. 3*B*). Immunoprecipitation with this antibody in basal conditions has been also shown for this technique (22) and could be related to a partial unspecificity in antibody detection. To assess this unspecificity, we treated the cell extracts with Hg^{2+} to break the nitrosothiol bonds, which reduced the specific immunoprecipitation after CSNO treatment, although maintaining an unspecific binding in basal or Cys-treated cells (Fig. 3*C*). These results confirm that Hsp90 is S-nitrosylated inside endothelial cells treated with CSNO and support the hypothesis that Hsp90 S-nitrosylation can be promoted when eNOS is activated.

S-Nitrosylation of Hsp90 Inhibits Intrinsic Hsp90 ATPase Activity and eNOS Positive Regulation. An inherent ATPase activity has been shown to be essential for Hsp90 to perform its correct function in the folding of several regulatory proteins (14, 23). We investigated whether S-nitrosylation of purified Hsp90 could alter this intrinsic property by performing ATPase assays, coupled to detection of NADH consumption as described in *Materials and Methods*. As shown in Fig. 4*A*, S-nitrosylation of Hsp90 promoted a significant inhibition of ATPase activity, which was comparable with the inhibition by the specific Hsp90 inhibitor, geldanamycin. Fig. 4*B* shows how S-nitrosylated Hsp90 follows a different slope compared with the native protein, reflecting a fundamental difference in the ability of the two proteins to hydrolyze ATP. Hsp90 treated with geldanamycin follows a similar pattern when compared with Hsp90 treated with GSNO.

Positive regulation of eNOS activity by Hsp90 is well established (24). Thus, it was important to address whether Hsp90 S-nitrosylation could influence this effect. We measured eNOS activity by quantifying the conversion of isotopically labeled L-arginine into L-citrulline because this reaction proceeds with the same stoichiometry as the conversion of the guanidino group of L-arginine into NO (25, 26) and to avoid potential interference of S-nitrosylated Hsp90 with systems evaluating NO synthesis directly (Fig. 5). Coincubation of purified eNOS with Hsp90 enhanced eNOS activity (≈50%) compared with eNOS alone, consistent with previous reports (24, 27). However, S-nitrosylated recombinant Hsp90 had no positive effect on eNOS activity (Fig. 5). When eNOS was incubated in the presence of S-nitrosylated GAPDH, no inhibitory effect on L-arginine to L-citrulline conversion was observed (data not shown).

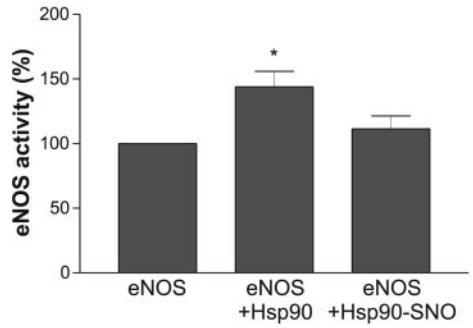

Fig. 5. eNOS activation by Hsp90 is inhibited by S-nitrosylation. Purified eNOS (1 μM) was incubated with or without native or S-nitrosylated Hsp90 (1 μM), and eNOS activity was determined from the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline. Data are means ± SEM, $n = 7$ experiments in duplicate; *, $P < 0.01$ with respect to eNOS alone.

Discussion

We herein describe the S-nitrosylation of Hsp90 and show that this modification inhibits the intrinsic ATPase activity of Hsp90 needed for its function as a chaperone protein and a coactivator of eNOS. We performed three kinds of experiments to show that purified Hsp90 is S-nitrosylated and have mapped a cysteine residue involved to the region that interacts with eNOS. We have also shown by two complementary approaches that Hsp90 is S-nitrosylated in intact endothelial cells by a S-nitrosylating agent and that it could also become S-nitrosylated after treatment with physiological activators of eNOS.

S-nitrosylation has gained increasing recognition as a functionally important posttranslational modification. Interest in NO signaling through the formation of covalent bonds (so-called "indirect effects" of NO) arises mainly from the ability of NO (and/or related reactive nitrogen species) to form physiologically labile nitrosothiol bonds. Added to this property is the essential role played by many reactive cysteine residues in signaling proteins and transcriptions factors (1, 2). Numerous examples of protein S-nitrosylation have been reported *in vitro* and *in vivo*. Work from several laboratories has established the biochemical conditions that favor S-nitrosylation and even found potential mechanisms for enzymatic regulation for S-denitrosylation; for recent reviews, see refs. 28–31. Two problems have prevented wider acceptance of S-nitrosylation as an essential mechanism for the regulation of protein function. Many demonstrations of S-nitrosylation have been on purified proteins and achieved with high concentrations of exogenous NO donors. Besides, there are few demonstrations of functional consequences or of the cellular occurrence of the modification. Although this study displays some of these limitations, our data provide strong evidence for the existence of specific S-nitrosylation *in vitro* and in endothelial cells. We used physiological S-nitrosylating agents for the *in vitro* studies, albeit at concentrations only encountered under inflammatory conditions. In addition, the promotion by the physiological eNOS activator VEGF of detectable S-nitrosylation in cell extracts suggests that S-nitrosylation of Hsp90 may indeed occur inside cells. This conclusion is consistent with the idea of the importance of subcellular colocalization of the target and nitrosylating agents. In the case of Hsp90, it is clear that the proximity of the target to a source of NO, eNOS, can favor its S-nitrosylation.

ATPase activity specifically inhibited by geldanamycin and radicicol, implied at least in some of the chaperone activities described for the protein, is well characterized in the N-terminal domain of Hsp90 (32), although a putative secondary ATP binding site has also been described to exist in the C-terminal domain (33–35). No cysteine is located in the N-terminal domain, and Cys 597, which we have identified as a target for S-nitrosylation, is located in the C-terminal domain, in a strand belonging to a β-sheet separated from the dimerization helices described to be involved in the putative secondary ATP-binding site (11). Thus, the detailed influence of Hsp90 S-nitrosylation (or other putative cysteine modifications mapped to the C-terminal domain, like cisplatin inhibition) on the ATP cycle of Hsp90 is not clear at first glance, but it would be related to the ability of regions far from the N-terminal domain to modulate its ATPase activity (36, 37).

Since its molecular characterization and cloning (38), the regulation of eNOS activity has revealed as being increasingly complex (3, 39–41). Several years ago, remarkable experimental work revealed that Akt and Hsp90 regulated eNOS activity (24, 42, 43). Furthermore, Akt-dependent phosphorylation and phosphatase activation are important for eNOS activation by growth factors or hormones (3, 40). It is now clear that both calcium-dependent and -independent mechanisms are operative and that protein–protein interactions are essential for both negative (caveolin-1) and positive (calmodulin, Hsp90) modulations. In this regard, S-nitrosylation of Akt has been recently described, adding one further step of complexity to modifications of eNOS regulatory proteins (44, 45).

In the original report proving the interaction of Hsp90 and eNOS, it was suggested that Hsp90 could act as an allosteric modulator of eNOS by inducing a conformational change in the enzyme or by stabilizing the dimeric form (24). A direct interaction between eNOS and Hsp90 has been demonstrated (27), and Hsp90 has been implicated in many of the positive regulatory mechanisms of eNOS, both Ca^{2+}-dependent and -independent (46–49). Two different studies report cooperativity between Hsp90 and Akt in relation to VEGF and insulin (50, 51). The data in these studies suggest that Hsp90 promotes the transition of eNOS-Ca^{2+} dependency to eNOS-Ca^{2+} independency by synergizing with Akt, thus lending a molecular basis for a calcium-independent operative mechanism. It has also been proposed that Hsp90 acts as a scaffold protein for eNOS and Akt, facilitating eNOS phosphorylation by Akt (19, 50). This hypothesis fits well with the biochemical and structural data available, which show that there are different $\alpha\beta\alpha$ structural motifs in the M domain of Hsp90 where the two proteins could interact without a structural overlapping (Fig. 2*B*; refs. 19, 52, and 53).

Data shown in this work locate a substrate residue for S-nitrosylation to the end of the region of Hsp90 originally described to interact with eNOS (19), although it lies in a separate structural motif from the main part of this region (Fig. 2*B*; refs. 11 and 53). Further detail of the interaction between eNOS and Hsp90 would reveal whether the region containing the S-nitrosylated residue participates directly in it. Whatever the structural detail, the data we show here point to the importance of the modification in inhibiting the activation of eNOS by Hsp90. S-nitrosylation of enzymes involved in arginine production (54), activation of eNOS (55), and eNOS itself (56, 57) has been recently reported. In the case of eNOS, it was shown that S-nitrosylation inhibits its enzymatic activity (57). It could be argued that, in our experiments, inhibition of eNOS activation by Hsp90 was due to a transnitrosylation to eNOS. However, the fact that S-nitrosylated GAPDH did not alter eNOS activity does not suggest such unspecific transnitrosylation. Rather, these data point to the possibility that S-nitrosylation of Hsp90 induces a conformational change that disrupts the interaction between eNOS and Hsp90. Recently, the role of NO-derived modifications in altering protein–protein interactions was reported (58); our results stress that this alteration can also happen in the NO producing system.

This work may have implications in two different conceptual areas. First, it provides a mechanism for limiting eNOS activity in a regulated manner through the inhibition of an interacting protein. Clearly, this limitation could represent a feedback

mechanism for situations where overproduction of NO might be harmful for the endothelial cell, such as shear stress. Alternatively, it could contribute to a cycle of eNOS activation/inactivation under physiological conditions, together with other crucial regulators such as caveolin and calmodulin (39). Second, Hsp90 has been recently implicated in tumor growth and cell invasiveness (7, 59), and Hsp90 inhibitors are being evaluated as therapeutic antitumor agents (60, 61). The fact that S-nitrosylation alters such a fundamental function of Hsp90 as its ATPase activity suggests that it might regulate these roles of the chaperone, perhaps explaining some of the antitumoral effects attributed to NO in several studies (62). The data reported here propose further complexity to our picture of eNOS regulation but also shed light on the role of S-nitrosylation of eNOS regulatory proteins and open perspectives on their mode of interaction.

We thank Estrella Soria for technical assistance and lab members for helpful discussions. This research has been supported by grants from the Plan Nacional de I+D+I, Ministerio de Ciencia y Tecnología (SAF 2000-0149, SAF 2003-01039, and BIO 2003-01805), Comunidad Autónoma de Madrid (08.4/0030.1/2003), and Ministerio de Sanidad y Consumo (RECAVA network and CP03/00025) and by an institutional grant by Fundación Ramón Areces to Centro de Biología Molecular "Severo Ochoa," all in Spain. A.M.-R. has received a fellowship from the Comunidad Autónoma de Madrid (cofinanced by the European Social Fund). L.V. is a recipient of a fellowship from the Fundación Carolina, C.G.d.O. from the Ministerio de Educación y Ciencia, and C.T. from the Fundación Renal "Íñigo Álvarez de Toledo," all in Spain.

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