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

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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 | nitration | 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 formation of higher nitrogen oxides (1, 2). To date, 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 NaNO2 in 0.5 M HCl (molar ratio Hsp90:NO2− 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 refs. 5 and 9, either in vitro 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 NO2− as above, subjected to cold acetone precipitation, lyophilized to dryness, and dissolved in 20 μl of...
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 LTO 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 MgCl₂, 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, Upppsala). 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 3H-labeled L-arginine to 3H-labeled L-citrulline was used to determine the activity of purified recombinant eNOS. Hsp90 was S-nitrosylated with NaN₂, as previously described, pH was neutralized, and S-nitrosylation was assessed by ozone chemiluminescence, giving a S-nitrosothiol content of ~1 mol per mol of Hsp90. Control Hsp90 was treated in parallel with acid in the absence of NaN₂. 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 CaCl₂, 10 μg/ml calmodulin, and 0.1 μCi (1 Ci = 37 GBq) 3H-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 NO₂⁻, the NO donor diethylamine-NONOate, or a nitrosothiol (GSNO). A clear band was detected when it was subjected to the biotin switch assay (Fig. 1B).

Treatment with Cu²⁺/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. 1B) or acidified NO₂⁻ (Fig. 1C). 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. 1B).

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 NO₂⁻ 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. 2A, 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 b¹⁺ ion (Fig. 2A), 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. 2B; 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. 2C) 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
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
we treated the cell extracts with Hg²⁺ unspecificity in antibody detection. To assess this unspecificity, shown for this technique (22) and could be related to a partial precipitation with this antibody in basal conditions has been also treated in total protein extracts and in eluates by SDS-PAGE and immunoblot. Hsp90 was identified by immunoblot. As shown in Fig. 3A, after the biotin switch assay, and the biotinylated proteins were purified with avidin-agarose, followed by elution with 2-mercaptoethanol. Hsp90 was detected in extracts from EA.hy926 cells untreated, treated with Cys or CSNO (1 mM) or exposed to 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. As shown in Fig. 3B, after the biotin switch assay, and the biotinylated proteins were purified with avidin-agarose, followed by elution with 2-mercaptoethanol. Hsp90 was identified by immunoblot. Figures shown are representative of at least two independent experiments.

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 Hg²⁺ and Hsp90 identified by immunoblot. Figures shown are representative of at least two independent experiments.

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. 3A, 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. 3B). 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. 3B). 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 Hg²⁺ 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. 3C). 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. 4A, S-nitrosylation of Hsp90 promoted a significant inhibition of ATPase activity, which was comparable with the inhibition by the specific Hsp90 inhibitor, geldanamycin. Fig. 4B 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).
Examples of protein S-nitrosylation have been reported. Alkylation labile nitrosothiol bonds. Added to this property is the in vivo 'indirect effects' of NO) arises mainly from the ability of NO actionally important posttranslational modification. Interest in NO agent and that it could also become S-nitrosylated after treat-
maintained Hsp90 is S-nitrosylated and have mapped a cysteine
domain of Hsp90 (32), although a putative secondary ATP
has been implicated in many of the positive regulatory mechanisms
of eNOS, both Ca\(^{2+}\)-dependent and -independent (46–49). Two
different studies report cooperation between Hsp90 and Akt in
relation to VEGF and insulin (50, 51). The data in these studies sug-
S-nitrosylation of Akt has been recently described, adding one further step of complexity to modific-
tions 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 sug-
S-nitrosylation 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 αβε structural motifs in the M domain of Hsp90 where the two proteins could interact without a structural overlapping (Fig. 2B; 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. 2B; 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

![Fig. 5](image-url)

**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 treat-
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**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.
propose further complexity to our picture of eNOS regulation attributed to NO in several studies (62). The data reported here indicate that nitrosylation alters such a fundamental function of Hsp90 as its chaperone, perhaps explaining some of the antitumoral effects of therapeutic antitumor agents (60, 61). The fact that S-nitrosylation has been recently implicated in tumor growth and cell inactivation under physiological conditions, together with other regulatory proteins and open perspectives on their mode of interaction.

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