Opposing Actions of Heat Shock Protein 90 and 70 Regulate Nicotinamide Adenine Dinucleotide Phosphate Oxidase Stability and Reactive Oxygen Species Production

Feng Chen, Yanfang Yu, Jin Qian, Yusi Wang, Bo Cheng, Christiana Dimitropoulou, Vijay Patel, Ahmed Chadli, R. Dan Rudic, David W. Stepp, John D. Catravas, David J.R. Fulton

Objective—Excessive reactive oxygen species contribute to vascular dysfunction. We have previously shown that heat shock protein (Hsp90) inhibitors potently suppress Nox 1 to 3 and 5, and the goals of this study were to identify how molecular chaperones regulate Nox function.

Methods and Results—In vitro, protein expression of Nox 1 to 2, 5 was decreased by Hsp90 inhibitors in multiple cell types (human pulmonary artery endothelial cells, neutrophils, macrophages, and human saphenous vein). In mice treated with Hsp90 inhibitors, Nox1 expression was reduced in lung along with reduced reactive oxygen species from leukocytes. Elevated reactive oxygen species production in obese (db/db) aorta was suppressed by Hsp90 inhibition. Hsp90 inhibitors did not alter Nox5 micro RNA levels, and proteasome inhibition prevented Nox2 and 5 protein degradation and increased ubiquitin incorporation. Inhibition of Hsp90 upregulated the expression of Hsp70 and Hsp70-bound Nox2, 5 and promoted degradation. Silencing Hsp70 prevented Hsp90 inhibitor–mediated degradation of Nox5. The Hsp70-regulated ubiquitin ligase, CHIP, also bound Nox5 and promoted increased Nox5 ubiquitination and degradation. The chaperone binding and ubiquitination domains of CHIP were required, and the silencing of CHIP blunted Hsp90 inhibitor–mediated degradation of Nox2 and 5.

Conclusion—We conclude that Hsp90 binds to and regulates Nox protein stability. These actions are opposed by Hsp70 and CHIP, which promote the ubiquitination and degradation of Nox proteins and reduce reactive oxygen species production. (Arterioscler Thromb Vasc Biol. 2012;32:XX-XX.)

Key Words: reactive oxygen species ■ NADPH oxidase ■ Hsp90 ■ Hsp70 ■ CHIP ■ inflammation ■ vascular biology

In the vasculature, reactive oxygen species (ROS), including superoxide (O_2^·), hydrogen peroxide (H_2O_2), and derivative oxygen radicals, are generated by the nicotinamide adenine dinucleotide phosphate oxidase (Nox) family of enzymes. Of the 7 Nox family members, vascular and myeloid cells express Nox1, 2, 4, and 5 (*Nox5* gene is not present in rat and mouse genomes). Although the importance of ROS to the obligate function of immune cells has been extensively defined, the physiological roles of the various Nox isoforms in the blood vessel wall remain largely enigmatic. What is well known is that cardiovascular diseases promote increased ROS production in blood vessels with the consequence of altered vaso-motor tone, compromised endothelial function, and increased vascular cell proliferation and migration.

Considerable insight has been gained into the posttranslational regulation of Nox enzymes. The mechanisms involved vary significantly according to Nox isoform and cell type. The amount of ROS produced can also be regulated through changes in the gene expression of Nox isoforms and subunits. Many vasoactive, prohypertensive, and proproliferative hormones have been shown to increase the expression of Nox proteins via activation of well-characterized transcriptional networks. In comparison, the mechanisms regulating the protein stability of the Nox isoforms are poorly understood. Previously we identified heat shock protein 90 (Hsp90) as a novel regulator of Nox5-dependent ROS production. Loss of Hsp90 function through genetic or pharmacological approaches poetically reduces Nox5-dependent ROS production. A chaperone-dependent function of Hsp90 in controlling the folding of Nox5 is further supported by the loss of Nox5 expression in the presence of Hsp90 inhibition.

Hsps are abundant proteins that function as molecular chaperones. Hsp90 regulates the function of ~200 identified client proteins by influencing their 3-dimensional structure and stability. The majority of Hsp90 clients are transcription factors, kinases, or other signaling molecules such as the NO synthases.
Inhibitors of Hsp90, such as radicicol (RAD), geldanamycin, and analogs (17-AAG, DMAG) occupy an N-terminal ATP-binding site on Hsp90 and impede client protein folding. Recently it has been shown that Hsp90 levels are elevated in both plaques and serum from individuals with atherosclerosis. Inhibition of Hsp90 has been shown to suppress inflammation and reduce ROS production in atherosclerosis, but the mechanisms involved are poorly understood. A consequence of reduced Hsp90 activity is increased expression of Hsp70, and the selective upregulation of Hsp70 itself has been shown to provide cardiovascular protection suggesting that Hsp70 might mediate some of the beneficial effects of Hsp90 inhibition.

Thus, the major goals of this study were to determine whether the expression of other Nox proteins is regulated by Hsp90, to identify the role of Hsp70 in this process, and to identify the mechanisms of Nox protein degradation.

### Materials and Methods

**Cell Culture**

COS-7, HEK293, and promyelocytic leukemia cells (HL-60) were grown in DMEM as described. Human pulmonary artery endothelial cells and human aortic smooth muscle cells were from Cascade Biologics and grown in EBM-2 or SmBM (Clonetics). Cells were transfected as described. HA-Nox5 cell line was generated by Flp recombinase-mediated integration (Invitrogen). Cells were exposed to different concentrations of RAD, 17-AAG (Fisher Scientific), geldanamycin (Biosciences), geranylgeranylacetone (GGA), and MG-132 (Sigma) for 0.5 to 24 hours. Bone marrow–derived macrophages from WT and Nox2–/– mice were obtained from Cell Biologics. Segments of intact human saphenous vein were obtained as discarded tissues. The procurement of these tissues conforms to the principles outlined in the Declaration of Helsinki and was approved by the Human Assurance Committee of the Georgia Health Sciences University.

**Mouse Macrophage Isolation and Animal Studies**

Male C57Bl/6 mice (7 to 10 weeks of age; Harlan, Indianapolis, IN) were used in all experiments. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at Georgia Health Sciences University. Mouse macrophages were isolated from the peritoneum after injection of thioglycolate. In brief, 1 mL of thioglycolate was injected into each mouse 3 to 5 days before collection, peritoneal cells were collected by lavage, red blood cells lysed with hypotonic buffer, and isolated macrophages were cultured in complete RPMI medium. Hsp90 inhibitors (17-AAG, dissolved in 10% DMSO, or 17-DMAG, dissolved in saline) were administered immunoprecipitated 16 to 24 hours before euthanization and isolation of lung tissue and blood.

**DNA and Adenoviral Constructs**

Plasmid DNA-coding Nox5 (AF325189), Nox1, Nox3, and Nox4 were described previously. The complementary DNAs for Nox5 and GAPDH (loading control).
Inhibitors of Hsp90, such as radicicol (RAD), geldanamycin, and analogs (17-AAG, DMAG) occupy an N-terminal ATP-binding site on Hsp90 and impede client protein folding. Recently it has been shown that Hsp90 levels are elevated in both plaques and serum from individuals with atherosclerosis. Inhibition of Hsp90 has been shown to suppress inflammation and reduce ROS production in atherosclerosis, but the mechanisms involved are poorly understood. A consequence of reduced Hsp90 activity is increased expression of Hsp70, and the selective upregulation of Hsp70 itself has been shown to provide cardiovascular protection suggesting that Hsp70 might mediate some of the beneficial effects of Hsp90 inhibition. Thus, the major goals of this study were to determine whether the expression of other Nox proteins is regulated by Hsp90, to identify the role of Hsp70 in this process, and to identify the mechanisms of Nox protein degradation.

Materials and Methods

Cell Culture

COS-7, HEK293, and promyelocytic leukemia cells (HL-60) were grown in DMEM as described. Human pulmonary artery endothelial cells and human aortic smooth muscle cells were from Cascade Biologics and grown in EBM-2 (Clonetics). Cells were transfected as described. HA-Nox5 cell line was generated by Flp recombinase-mediated integration (Invitrogen). Cells were exposed to different concentrations of RAD, 17-AAG (Fisher Scientific), geldanamycin (Biosciences), geranylgeranylacetone (GGA), and MG-132 (Sigma) for 0.5 to 24 hours. Bone marrow–derived macrophages from WT and Nox2–/– mice were obtained from Cell Biologics. Segments of intact human saphenous vein were obtained as discarded tissues. The procurement of these tissues conforms to the principles outlined in the Declaration of Helsinki and was approved by the Human Assurance Committee of the Georgia Health Sciences University.

Mouse Macrophage Isolation and Animal Studies

Male C57Bl/6 mice (7 to 10 weeks of age; Harlan, Indianapolis, IN) were used in all experiments. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at Georgia Health Sciences University. Mouse macrophages were isolated from the peritoneum after injection of thioglycolate. In brief, 1 mL of thioglycolate was injected into each mouse 3 to 5 days before collection, peritoneal cells were collected by peritoneal lavage with red blood cell lysis buffer, and isolated macrophages were cultured in complete RPMI medium. Hsp90 inhibitors (17-AAG, dissolved in 10% DMSO, or 17-DMAG, dissolved in saline) were administered immunoprecipitated 16 to 24 hours before euthanization and isolation of lung tissue and blood.

DNA and Adenoviral Constructs

Plasmid DNA-coding Nox5β (AF325189), Nox1, Nox3, and Nox4 were described previously. The complementary DNAs for myc-ChIP, myc-ChIP H260Q, and myc-ChIP K30A were gifts from Dr Cam Patterson (University of North Carolina). Other cDNAs were obtained from Origene or Addgene. Control (RFP) and HA-Nox5 adenoviruses have been described.

Comunoprecipitation and Western Blotting Analysis

Cells were lysed on ice in 20-mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 100-mmol/L NaCl, 1-mmol/L NaVO4, 10-mmol/L NaF, and 1% protease inhibitor cocktail (Sigma). Soluble extracts were incubated for 2 hours at 4°C with relevant antibodies: anti-HA (Roche Applied Science), anti-Nox2 (Sigma), anti-ubiquitin (Cell Signaling Technology), and a negative isotype control mouse immunoglobulin (Santa Cruz Biotechnology), and complexes precipitated with protein A/G agarose (Santa Cruz Biotechnology). Western blotting was performed as described previously using anti-HA (Roche), anti-Nox1, 2 (Sigma), anti-Hsp90, myc (BD Biosciences), anti-ubiquitin, anti-GFP, Hsp70 (Cell Signaling Technology), and anti-CHIP.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction was performed as described using human Nox5β (5'-cctcttagttgcttgc-3' (sense) and 5'-tagggaccactgctc-3' (antisense), human 18S: 5'-Cagccaccgagattgagca-3' (sense) and 5'-tagggaccactgctc-3' (antisense). The measurements of superoxide (L-012) and hydrogen peroxide (Amplex Red) have been described. 17,18,20,22 2-Hydroxyethylidium (2-OH-E+), the specific product of the reaction of hydroethidine with superoxide radical anion (O2−), was measured in HL-60 cells as described elsewhere with recent modifications. Tissue dihydroethidium staining was performed as described. In brief, mouse aortas were treated with vehicle or RAD 20 µmol/L and embedded in OCT, sectioned, and incubated with dihydroethidium (5 µmol/L, Invitrogen).

Transient Knockdown of CHIP Gene With Small Interfering RNA

The with small interfering RNA (siRNA)-targeting CHIP (STUB1, siRNA ID: s195025) was obtained from Applied Biosystems. Validated control and targeting siRNA were transfected into HEK293 cells stably expressing Nox3 and HL-60 cells using siPORT Amine (Applied Biosystems).

Statistical Analysis

Data are reported as mean±SE, and statistical analyses were performed using Instat and a 2-tailed Student t test or ANOVA with a post hoc test where appropriate. Differences were considered as significant at P<0.05.

Results

Hsp90 Is Necessary for the Maintenance of Nox Protein Stability

To determine the effect of Hsp90 inhibition on the stability of other Nox family members, we incubated COS-7 cells
expressing Nox5, human pulmonary artery endothelial cells, murine macrophages, a human neutrophil cell line (HL-60), and intact segments of human saphenous vein with Hsp90 inhibitors. We found that prolonged inhibition of Hsp90 robustly decreased the expression of Nox5 in COS-7 cells, Nox2 in macrophages and neutrophils, Nox1 and Nox2 in human pulmonary artery endothelial cells, and Nox1, 2, and 5 in human saphenous vein (Figure 1A–1E). Expression of GAPDH was not altered; densitometry for Figure 1D and 1E is shown in Figure I in the online-only Data Supplement. Hsp90 inhibitors reduced superoxide levels in WT macrophages but did not influence the dramatically reduced levels in Nox2−/− mice (Figure 1B, right). Hsp90 reduced superoxide levels in PMA-stimulated neutrophils as determined by 2-OH-E+ levels (Figure 1C, right). In vivo, exposure of mice to the Hsp90 inhibitor 17-DMAF for 24 hours resulted in reduced Nox1 expression, but not Nox4, in the lung (Figure 1F; densitometry for Figure 1F is shown in Figure I in the online-only Data Supplement). In leukocytes isolated from mice treated with 17-AAG for 16 hours, there was a significantly reduced capacity to generate superoxide (Figure 1G).

The upregulation of Hsp70 in leukocytes from mice treated with 17-AAG is indicative of Hsp90 inhibition (Figure I in the online-only Data Supplement). Aortas from obese db/db mice exhibit higher dihydroethidium oxidation levels in the media and adventitia versus that in lean mice (Figure 1H; Figure II in the online-only Data Supplement), and this was reduced in the presence of Hsp90 inhibitors.

**Hsp90 Inhibition Promotes the Ubiquitination and Proteasomal Degradation of Nox5 and Nox2**

To exclude the possibility that inhibition of Hsp90 decreases Nox5 protein expression via effects on CMV promoter activity or micro RNA levels, we first generated an additional expression vector. We found that RAD effectively decreased Nox5 protein expression driven by both CMV and EF-1α promoters and did not alter micro RNA levels (Figure II in the online-only Data Supplement; Figure 2A). Inhibition of Hsp90 did not influence the protein expression of p22phox (Figure II in the online-only Data Supplement). Confirmation of the effect of Hsp90 inhibitors on Nox protein stability was derived from the ability of the proteasome inhibitor, MG-132 to block the loss of Nox5 and Nox2 expression in the presence of the Hsp90 inhibitors, RAD (Figure 2B and 2C) and geldanamycin (Figure III in the online-only Data Supplement). Because these data suggest the involvement of the ubiquitin-proteasome pathway, we next examined whether ubiquitin overexpression can promote Nox5 degradation. In cells transfected with ubiquitin and Nox5, we observed significant loss of Nox5 protein compared with cells in control (Figure 2D). To determine whether Nox5 is ubiquitin labeled, cells were treated with or without geldanamycin in the presence of MG-132, and ubiquitin-labeled proteins were isolated by immunoprecipitation. We observed increased ubiquitin-labeled Nox5 in the presence of Hsp90 inhibition (Figure 2E). These results were confirmed by reciprocal experiments immunosolating Nox5 and blotting for ubiquitin (Figure 2F and 2G).

**Hsp70 Physically Associates With Nox and Is Important for Nox Activity and Stability**

Inhibition of Hsp90 resulted in the upregulation of Hsp70 in vitro in cells (Figure 3A) and in vivo (Figure I in the online-only Data Supplement). To determine whether Hsp70 is important for Nox5 regulation, we first tested whether Nox5 is a client protein of Hsp70 using coimmunoprecipitation. We found that Hsp70 physically associates with Nox5 (Figure 3B). To investigate the effect of Hsp70 on Nox5 activity and protein stability in the absence of Hsp90 inhibition, we increased Hsp70 levels by cotransfection. Increased Hsp70 decreased Nox5 activity and promoted Nox5 degradation (Figure 3C). GGA, a nontoxic antiulcer drug, has been shown to acutely increase Hsp70 expression. We exposed cells expressing Nox5 to GGA and found that upregulation of Hsp70 decreased Nox5-derived superoxide in a time- and dose-dependent manner (Figure 3D; Figure III in the online-only Data Supplement). Cells treated with GGA exhibited increased binding of Hsp70 to Nox5 (Figure 3E). We also found that Hsp70 upregulation decreased Nox1- and Nox3-derived superoxide, but not Nox4-derived hydrogen peroxide production (Figure IV in the online-only Data Supplement). To determine whether Hsp70 is necessary for Nox5 degradation in response to Hsp90 inhibition, we silenced Hsp70 using a siRNA approach. In cells with diminished Hsp70 levels, there was a reduced ability of Hsp90 inhibitors to decrease Nox5 expression (Figure 3F).

**CHIP Associates with Nox5**

In the presence of Hsp90 inhibitors, we observed increased binding to Hsp70 and diminished binding to Hsp90 revealing a reciprocal pattern of chaperone-dependent regulation (Figure 4A). In addition to Nox5, we also examined whether this pathway was operational in cells expressing Nox2 endogenously. Human peripheral blood mononuclear cells were treated with RAD and Nox2 was immunoprecipitated. Inhibition of Hsp90 resulted in reduced amounts of Hsp90 bound to Nox2 and increased Hsp70 binding (Figure 4B). We next assessed whether the Hsp70-interacting protein (CHIP), previously identified as a regulator of protein quality-control of Hsp90-dependent client proteins, binds to Nox proteins. CHIP has a tetratricopeptide repeat (TPR) domain at its N terminus, which interacts with Hsp70, and a U-box domain at its C terminus, which contains an E3 ubiquitin ligase. To examine whether CHIP physically associates with Nox5, cells were cotransfected with HA-Nox5 and myc-tagged CHIP wild type or mutants: CHIP H260Q (a U-box domain mutant) and CHIP K30A (a TPR domain mutant). Using a Co-IP approach, we found a strong association between both the wild-type and the E3 ubiquitin ligase–deficient CHIP mutant (H260Q), whereas CHIP lacking an intact TPR domain did not associate with Nox5 (Figure 4C). These data suggest that CHIP incorporation into Nox complexes is through interaction with the TPR recognition motif of Hsp70.

**CHIP Induces the Degradation of Nox5 and Reduces Superoxide Production**

Increased expression of CHIP, but not either of the CHIP mutants, dose-dependently decreased Nox5-dependent ROS
production and protein expression (Figure 5A–F). To broaden the functional significance of CHIP in regulating the activity and stability of other Nox isoforms, we transfected CHIP together with Nox1, Nox3, and Nox4 and found that CHIP decreased the activity of Nox1 and Nox3, whereas ROS production from Nox4 was unaffected (Figure IV in the online Data Supplement). The increased expression of wild-type CHIP promoted Nox5 ubiquitination, an effect that was absent in cells expressing either of the CHIP mutants (Figure 5G). To determine for the role of endogenous CHIP in the degradation of Nox proteins, we silenced CHIP expression using siRNA (Figure 6A). Reduced expression of CHIP abrogated the degradation of Nox5 induced by Hsp90 inhibitors (Figure 6B and 6C). Further, the ability of Hsp90 inhibitors to induce Nox5 ubiquitination was reduced in cells with diminished levels of CHIP (Figure 6D and 6E). To determine whether CHIP is necessary to mediate the effects of increased Hsp70 expression on Nox5 activity, we silenced CHIP and induced Hsp70 expression in cells expressing Nox5. In cells treated with control siRNA, GGA decreased the ability of Nox5 to generate superoxide. In contrast, cells treated with CHIP siRNA reduced the expression levels of CHIP, and superoxide production was unaffected by GGA treatment (Figure 6F). We also investigated whether CHIP can regulate the expression of other Nox enzymes. In a human neutrophil cell line, silencing CHIP reduced the ability of Hsp90 inhibition to decrease endogenous Nox2 expression (Figure 6G). In addition to CHIP, there are multiple chaperone-associated E3 ubiquitin ligases, including Cullin5, valosin-containing protein, Parkin, and MDM2 that are stimulated by the inhibition of Hsp9027-30.

To determine whether additional E3 ubiquitin ligases regulate Nox5 activity and stability, cells expressing Nox5 were transfected with plasmids encoding Cullin5, valosin-containing protein, Parkin, and MDM2 or siRNA to Cullin5 in the presence of Hsp90 inhibition. None of these ligases had a pronounced effect on Nox5 activity or protein expression, suggesting that they are not the ubiquitin ligases that target Nox5 (Figure V in the online-only Data Supplement).
Figure 3. Heat shock protein 70 (Hsp70) binds and is necessary and sufficient to regulate nicotinamide adenine dinucleotide phosphate oxidase (Nox5) activity and protein stability. A, COS-7 cells expressing Nox5 were treated with or without radicicol (RAD) (20 μmol/L) for 12 h, and lysates were immunoblotted with anti-HA, Hsp70, and GAPDH antibodies. B, COS-7 cells expressing Nox5 were lysed and immunoprecipitated using either control immunoglobulin (IgG) or anti-HA antibody. Immune complexes were immunoblotted for HA-Nox5 or Hsp70. C, Superoxide release from COS-7 cells cotransfected with HA-Nox5 and either control (RFP) or progressively higher amounts of Hsp70-GFP. The relative expression of Nox5, Hsp70 vs GAPDH, was determined by Western blot. D, Human aortic vascular smooth muscle cells (HASMC) expressing Nox5 (MOI of 20) were treated with geranylgeranylacetone (GGA) at the indicated time points, and unstimulated superoxide release and relative expression of Hsp70 and HA-Nox5 were determined. E, HEK cells expressing Nox5 and either a control small interfering RNA or Hsp70 siRNA were exposed to vehicle or RAD (20 μmol/L) for 12 h, and lysates were probed for expressions of HA-Nox5, Hsp70, and GADPH. F, GGA increases HSP70 binding to Nox5. HASMCs were treated with GGA (30 μmol/L) for 4 h, and Nox5 immunoprecipitated and immune complexes blotted for HA-Nox5 and Hsp70. Data are expressed as mean±SE, *P<0.05 vs control. (n=4–6). IP indicates immunoprecipitation.

Discussion

Hsp90 and cochaperones regulate the tertiary structure of select client proteins such as nuclear receptors, kinases, and NO synthases to enable specific enzyme functions. We have previously shown that the binding of Hsp90 to Nox5 regulates ROS production, and the current study shows that the protein expression of Nox1, Nox2, and Nox5 depends on active Hsp90. Inhibition of Hsp90 both in vitro and in vivo reduced...
Chen et al. Reciprocal Regulation of Nox Stability by Hsp70 and Hsp90

Nox expression and ROS production in isolated leukocytes, lung tissue, human blood vessels, and aorta from db/db mice. These results support the hypothesis that Hsp90 is integral to the function and stability of the Nox family of enzymes.

One exception to this paradigm was the inability of Hsp90 inhibitors to alter Nox4 expression and activity. We have previously found that the C-terminal region of Nox5 mediates the binding to Hsp90, and in domain-swapping experiments we have shown that the equivalent region in Nox4 is sufficient to provide resistance to Hsp90 inhibitors in Nox enzymes that are otherwise sensitive. Expression levels of p22phox can influence the stability of Nox proteins, but the inability of p22phox to influence Nox5, which is sensitive to Hsp90 inhibitors and the lack of effect of Hsp90 inhibitors on p22phox levels, suggest that indirect regulation via p22phox is not a major mechanism. The insensitivity of Nox4 to Hsp90 inhibition suggests that functions conferred by Hsp90 binding to other Nox enzymes are not needed for Nox4. Indeed, Nox4 has enzymatic characteristics unique from the other family members. It is regarded as constitutively active and emits only detectable hydrogen peroxide instead of superoxide. The mechanisms by which Hsp90 supports the stimulus-driven activity of Noxes 1, 2, 3, and 5 and the release of superoxide remain to be determined.

The upregulation of Hsp70 is a well-described outcome of Hsp90 inhibition and is mediated by the loss of Hsp90-dependent repression of HSF1. To delineate a distinct role for Hsp70 in the absence of Hsp90 inhibition, we increased Hsp70 levels via transfection of a GFP-tagged transgene. We found that the elevation of Hsp70 expression alone reduced ROS production from Nox5 and decreased Nox5 expression. These results suggest a direct interaction between Hsp70 and Nox5, and in Co-IP experiments we indeed found that Hsp70 binds to Nox5. This is the first evidence that Hsp70 is an essential component of Nox complexes. In addition to Nox5, we also found that Hsp70 decreases ROS production from Nox1 and Nox3, but not Nox4, and that Hsp70 binds to Nox2. These results further suggest that Hsp70 is a negative regulator of other Nox enzymes, and there is considerable evidence in the literature to support the protective role of Hsp70 against oxidative damage and the ability to negatively regulate Nox enzyme stability, and ROS production may contribute, at least in part, to the beneficial effects of Hsp70. These data are supported by previous studies showing that chemical or thermal induction of Hsps in neutrophils reduces ROS production. Mitogens and the protein kinase C agonist, PMA, have been shown to activate Noxes 1, 2, 3, and 5, and the ability of these stimuli to upregulate Hsp70 would also provide a mechanism for feedback inhibition. This feedback inhibition can be taken further because ROS themselves are potent inducers of Hsp70 expression, which would promote a reduction in nicotinamide adenine dinucleotide phosphate-dependent ROS production.

Protein quality control is a vital process that determines whether proteins are properly folded and targets misfolded proteins for degradation. Molecular chaperones, including Hsp90, Hsp70, and cochaperones, are important contributors.
Hsp90 regulates the folding and function of >200 known client proteins, and inhibition of Hsp90 promotes the degradation of many of these clients, including Akt, PDK, NO synthases, glucocorticoid receptor, sGC, and CFTR. Decreased function of Hsp90 results in the upregulation of Hsp70, which recruits specialized chaperone-directed ubiquitin ligases to promote client protein ubiquitination and degradation. Our current study shows that both Hsp90 and Hsp70 bind to Nox5, suggesting distinct roles for Hsp90 and Hsp70 in the regulation of Nox protein activity and stability. In the presence of Hsp90 inhibitors, we found a decreased association between Nox5 and Hsp90 and increasing binding to Hsp70. These data indicate that the ratio of Hsp90 and Hsp70 bound to Nox proteins is a critical mechanism regulating ROS production. Disruption of the Hsp90:Nox interaction or facilitation of the Hsp70:Nox interaction may be an effective strategy to suppress Nox-derived ROS and might have a clinical benefit in cardiovascular disease and inflammation.

The results of our study also indicate that ubiquitination is a novel and important posttranslational modification of Nox5 and that the ubiquitin-proteasome pathway regulates Nox5 degradation. The ubiquitination of proteins is orchestrated by ≤3 types of enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. The E3 ubiquitin ligases catalyze the transfer of

**Figure 5.** CHIP promotes nicotinamide adenine dinucleotide phosphate oxidase (Nox5) ubiquitination and subsequent degradation.

A–C, COS-7 cells were cotransfected with HA-Nox5 and increasing amounts of CHIP WT, CHIP H260Q, or CHIP K30A, and unstimulated superoxide release was determined by L-012 chemiluminescence. D–F, Cell lysates were immunoblotted for relative levels of Nox5, myc-CHIP, and GAPDH. G, COS-7 cells were cotransfected with HA-Nox5, GFP-ubiquitin and CHIP WT, CHIP H260Q, or CHIP K30A, and were exposed to MG-132 (5 μg/mL) for 12 h. Cell lysates were immunoprecipitated using anti-HA antibody and immunoblotted with anti-GFP or anti-HA antibodies. Results are representative of ≤3 separate experiments. Data are expressed as mean±SE, *P<0.05 vs vehicle (n=4–6).
ubiquitin to substrate proteins which are then recognized by the proteasome and degraded. The E3 ubiquitin ligases are a large and diverse family of enzymes and there are several, including CHIP, Cullin5, valosin-containing protein, Parkin, and MDM2 that have been shown to be regulated by molecular chaperones.27–30 The best characterized of these, CHIP, possesses a TPR domain at its N terminus, which interacts with molecular chaperones Hsp70/90, and a U-box.

Figure 6. Silencing CHIP ameliorates the loss of nicotinamide adenine dinucleotide phosphate oxidase (Nox) protein stability and increased ubiquitination induced by heat shock protein 90 (Hsp90) inhibition or induction of Hsp70. HEK cells stably expressing Nox5 were transfected with negative control small interfering RNA (siRNA) or siRNA-targeting CHIP, for 48 h. Vehicle or progressively higher doses of radicicol (RAD) were added to cells for 12 h in the presence and absence of MG-132 (5 μg/mL). A, Lysates were immunoblotted for CHIP. B, HA-Nox5 and GAPDH. C, Relative densitometry of Nox5 vs GAPDH expression. D, HEK cells stably expressing Nox5 were transfected with control siRNA or CHIP siRNA, and cells were treated with or without RAD for 12 h. Nox5 was immunoprecipitated using an anti-HA antibody and immunoblotted for Nox5 and ubiquitin. E, Relative densitometry of Nox5 ubiquitination. Data are expressed as percentage of control (means±SE, n=3, *P<0.05 vs Vehicle). F, HEK-Nox5 cells were transfected with scrambled or CHIP siRNA and exposed to GGA (0–50 μmol/L) for 4 h, and basal superoxide levels were measured with L-012. G, Neutrophil-differentiated HL-60 cells were transfected with scrambled or CHIP siRNA and were exposed to vehicle or RAD (5–20 μmol/L) for 16 h, and cell lysates were immunoblotted for Nox2, CHIP, Hsp70, and GAPDH. Results are representative of ≤3 separate experiments.
domain at its C terminus, which contains an E3 ubiquitin ligase domain. Both the TPR and U-box domains of CHIP are necessary for the degradation of Hsp90 client proteins. However, CHIP does not promote the degradation of all clients of Hsp90. It promotes the partitioning of endothelial NO synthase from a soluble to an insoluble and inactive cellular compartment, and more surprisingly, in fibroblasts, it can increase the refolding of proteins via its TPR domain. We found that CHIP associates in a complex with Nox5 and regulates the activity and stability of Nox5 and Nox2. Both the TPR and U-box domains of CHIP are necessary to induce the degradation of Nox5, which suggests that this process is chaperone dependent. Other chaperone-dependent E3 ligases, Cul5, valosin-containing protein, Parkin, and MDM2 did not appear to play a major role in the regulation of Nox protein stability. Collectively, these data demonstrate that chaperone and CHIP-dependent ubiquitination is a novel mechanism regulating Nox activity and stability.

Despite significant advances in the therapeutic approach, cardiovascular diseases remain the leading cause of morbidity and mortality in the United States. Elevated Hsp90 levels in individuals with atherosclerosis would be supportive of increased Nox activity and ROS production and contrast from the known protective effects of Hsp70 against oxidative damage and ROS levels. We found that altering the balance of Hsp90 to Hsp70 bound to Nox5 could alter protein stability and ROS production. In blood vessels from animal models of obesity and type II diabetes mellitus, the balance of Hsp90 to Hsp70 bound to Nox5 could alter oxidative damage and ROS levels. We found that altering the ratio of Hsp90 and Hsp70 bound to Nox5 and Nox2. The ratio of Hsp90 and Hsp70 bound to Nox5 is a critical mechanism regulating the proper folding and ROS production and guides Nox protein triage and stability of Nox proteins via the molecular chaperones, Hsp90 and Hsp70.

Conclusions
This study reveals a novel mechanism regulating the activity and stability of Nox proteins via the molecular chaperones, Hsp90 and Hsp70. The ratio of Hsp90 and Hsp70 bound to Nox proteins is a critical mechanism regulating the proper folding and ROS production and guides Nox protein triage decisions. Accordingly, disruption of the Hsp90:Nox interaction or facilitation of Hsp70:Nox binding might be an effective strategy to suppress ROS. We have also identified ubiquitination as a novel mechanism and an important posttranslational modification of Nox proteins that is regulated, at least in part, by the molecular chaperone and cochaperone CHIP. Further studies are necessary to elucidate the significance of this pathway in disease conditions.

Acknowledgments
We appreciate the contributions of Drs William O’Brien and Jacek Zielonka in the measurement of superoxide anion radical via 2-OH-E+ in HL-60 cells.

Sources of Funding
This work was supported by the National Institutes of Health grants R01 HL085827, P01 HL101902-01A1 (to D.J.R.F.), R01 HL092446 (to D.J.R.F. and D.W.S.), and by an established investigator award from the American Heart Association (to D.J.R.F.).

Disclosures
None.

References
heat shock protein 70 gene protects cardiac function against ischemia-reperfusion injury. 


Chen F, Fulton DJ. An inhibitor of protein arginine methyltransferases, 7,7’-carbonylbis(azanediyl)bis(4-hydroxyphenylacetamide)-2-sulfonic acid (AMI-1), is a potent scavenger of NADPH-oxidase-derived superoxide. 


Jagnandan D, Church JE, Banfi B, Stuehr DJ, Marrero MB, Fulton DJ. Novel mechanism of activation of NADPH oxidase 5. calcium sensitization via phosphorylation. 


Lo D, Dai MS, Sun XX, Zeng SX, Lu H. Ubiquitin- and MDM2 E3 ligase-independent proteasomal turnover of nucleostemin in response to GTP depletion. 


Durcan TM, Kontogianni ME, Bednar N, Wing SS, Fon EA. Ataxin-3 deubiquitination is coupled to Parkin ubiquitination via E2 ubiquitin-conjugating enzyme. 


Behnam K, Murray SS, Brochmann EJ. Identification and characterization of valosin-containing protein (VCP/p97) in untransformed osteoblast-like cells. 


Nisimoto Y, Jackson HM, Ozawa H, Karasawa T, Lambeth JD. Constitutive NADPH-dependent electron transferase activity of the Nox4 dehydrogenase domain. 


Jacquier-Sarin MR, Jornot L, Polla BS. Differential expression and regulation of hsp70 and hsp90 by phorbol esters and heat shock. 


Madamanchi NR, Li S, Patterson C, Range MS. Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. 


Theodoraki MA, Caplan AJ. Quality control and fate determination of Hsp90 client proteins. 


Basso AD, Solit DB, Chiosis G, Biris T, Tschilis P, Rosen N. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. 


Xu W, Marea M, Yuan K, Mimnaugh E, Patterson C, Nechkers L. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for unfolded Hsp90 client proteins. 


Jiang J, Cyr D, Babbitt RW, Sessa WC, Patterson C. Chaperone-dependent regulation of endothelial nitric-oxide synthase intracellular trafficking by the co-chaperone ubiquitin ligase CHIP. 


Libby P. Inflammation in atherosclerosis. 


Jacquier-Sarin MR, Jornot L, Polla BS. Differential expression and regulation of hsp70 and hsp90 by phorbol esters and heat shock. 


Madamanchi NR, Li S, Patterson C, Range MS. Reactive oxygen species regulate heat-shock protein 70 via the JAK/STAT pathway. 


Theodoraki MA, Caplan AJ. Quality control and fate determination of Hsp90 client proteins. 


Opposing Actions of Heat Shock Protein 90 and 70 Regulate Nicotinamide Adenine Dinucleotide Phosphate Oxidase Stability and Reactive Oxygen Species Production
Feng Chen, Yanfang Yu, Jin Qian, Yusi Wang, Bo Cheng, Christiana Dimitropoulou, Vijay Patel, Ahmed Chadli, R. Dan Rudic, David W. Stepp, John D. Catravas and David J.R. Fulton

Arterioscler Thromb Vasc Biol, published online September 27, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/09/27/ATVBAHA.112.300361

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/09/27/ATVBAHA.112.300361.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure I. (A) Densitometry analysis of Nox1, Nox2 and Nox4 in human pulmonary artery endothelial cells (HPAEC) in the presence and absence of vehicle, RAD(10-20µM) for 24h. (B) Densitometry analysis of Nox1, Nox2 and Nox5 in human saphenous vein in the presence and absence of vehicle, RAD(10-20µM). (C) Densitometric analysis of Nox1 and Nox4 in mice lung treated with vehicle or the Hsp90 inhibitor 17-DMAG (40mg/kg) in saline. (D) Blood was isolated from C57BL/6 mice treated with vehicle or the Hsp90 inhibitor 17-AAG (10mg/kg for 16h) and expression of Hsp70 in leukocytes determined by Western blotting. Data is expressed as means ± S.E.. *P<0.05 versus vehicle (n=6).

Supplemental Figure II. (A) DHE staining in lean (C57BL/6) mice in the presence and absence of RAD(20µM). (B) Loss of Nox5 protein expression is independent of the promoter, with CMV (upper panel) and (EF-1α) driven expression of Nox5 equally suppressed by RAD. Data is expressed as means ± S.E. *P<0.05 versus lacZ control (n=3-6). (C) Expression of p22Phox in HPAEC in the presence of RAD(10-20µM).

Supplemental Figure III. (A) COS-7 cells expressing Nox5 were treated with or without Geldanamycin (GA, 1µM, C), MG-132 (5µg/ml) (MG-132 was added 0.5 hr ahead), and cell lysates were collected after 12h and analyzed by Western blot. (B) Human aortic vascular smooth muscle cells (HASMC) expressing Nox5 (MOI of 20) were treated with increasing doses of geranylgeranylacetone (GGA, 0-50µM) for 2h and the relative production of superoxide was determined using L-012. Data is expressed as means ± S.E., n=6. *P<0.05 versus vehicle.

Supplemental Figure IV. Hsp70 and CHIP regulate Nox1 and Nox3 activity, but not Nox4. (A) Unstimulated superoxide release from COS-7 cells co-transfected with Nox1 and Hsp70, (B) Nox1 and CHIP, (C) Nox3 and Hsp70 and (D) Nox3 and CHIP were determined by L-012 chemiluminescence. (E) Unstimulated hydrogen peroxide release from COS-7 cells co-transfected with Nox4 and LacZ or Hsp70 or CHIP was measured using Amplex red fluorescence. Data is expressed as means ± S.E., n=6. *P<0.05 versus lacZ control (n=6).
Supplementary Figure V. E3 ubiquitin ligases, Cullin5, VCP, MDM2 and Parkin fail to decrease Nox5 activity and stability. (A) COS-7 cells were cotransfected with Nox5 and increasing amount of Cullin5 and treated with or without RAD for 12h. Cell lysates were immunoblotted for HA-Nox5. (B) HEK cells stably expressing Nox5 were transfected with control siRNA or Cullin5 siRNA for 48h, and lysates immunoblotted for HA-Nox5 versus GAPDH. (C) Cells expressing Nox5 was transfected with control plasmid RFP or valosin containing protein (VCP) and VCP mutants. Cell lysates were immunoblotted for HA-Nox5 versus GAPDH. (D) COS-7 cells were cotransfected with Nox5 and RFP or mouse double minute 2 (MDM2) and Parkin and superoxide production was determined by L-012 chemiluminescence. Data is expressed as means ± S.E., n=3-6.
A. Densitometry for HPAEC (Figure 1D)

Nox1

Nox2

Nox4

B. Densitometry for HSV (Figure 1E)

Nox1

Nox2

Nox5

C. Densitometry for mouse lung (Figure 1F)

Nox1

Nox4

D. Densitometry for mouse blood (Figure 1G)

Supplemental Figure I
A

Lean mice

DHE

Collagen

Overlay

Vehicle

RAD 20µM

B

IB: HA-Nox5

IB: GAPDH

P_{ef-1\alpha}-Nox5

IB: HA-Nox5

IB: GAPDH

P_{env}-Nox5

IB: HA-Nox5

IB: GAPDH

Vehicle

RAD

C

HPAEC

IB: p22

IB: GAPDH

Vehicle

RAD 10µM

RAD 20µM

Supplemental Figure II
Supplemental Figure III

(A) Relative expression of Nox5 relative to GAPDH. IB: HA-Nox5, IB: GAPDH. GA: - + - +, MG-132: - - + +.

(B) Superoxide Production RLU x10^3 at 2 hrs. GGA: 0 10μM 30μM 50μM.
**Supplemental Figure IV**

(A) Nox1

(B) Nox1

(C) Nox3

(D) Nox3

(E) Nox4

Superoxide Production

Hydrogen Peroxide Production

*RLU*10^3
Supplemental Figure V