Opposite Effect of Hsp90 α and Hsp90 β on eNOS Ability to Produce Nitric Oxide or Superoxide Anion in Human Embryonic Kidney Cells

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Key Words

eNOS phosphorylation • Monomer/dimer ratio • AKT/PKB • eNOS dimerization

Abstract

Heat shock protein 90 subfamily is composed by two cytosolic isoforms known as Hsp90 α and Hsp90 β . Endothelial nitric oxide synthase (eNOS) is regulated by Hsp90, however the specific role of each Hsp90 isoform on NO production has not been established. This study was designed to evaluate the effect of Hsp90 α and Hsp90 β over-expression on eNOS/NO pathway. Rat Hsp90 α and Hsp90 β were cloned into pcDNA3.1(+) and transfected in human embryonic kidney cells (HEK-293). Hsp90 α and Hsp90 β transfection was corroborated by Western blot analysis and their effect on NO production (NO₂/NO₃), eNOS protein and its phosphorylation at Ser1177 and Thr495, as well as Akt/PKB Ser473 phosphorylation was determined. The interaction of Hsp90 α and Hsp90 β with eNOS and the dimer/monomer ratio of Hsp90, as well as O₂⁻ generation were also assessed. After transfection, Hsp90 α and Hsp90 β levels were significantly increased in HEK-293 cells. The Hsp90 α over-expression induced a significant increase in NO₂/

NO₃ levels, an effect that was associated with increased phosphorylation of eNOS Ser 1177 and Akt/PKB Ser473, as well as with a greater Hsp90 α dimerization. Noteworthy, pcHsp90 β transfection reduced significantly NO₂/NO₃ and increased O₂⁻ generation. These effects were associated with a reduction of eNOS dimeric conformation, increased eNOS Thr495 phosphorylation, reduced Akt/PKB phosphorylation, and by a greater amount of monomeric Hsp90 β conformation. These data show for first time that Hsp90 α and Hsp90 β differentially modulate NO and O₂⁻ generation by eNOS through promoting changes in eNOS conformation and phosphorylation state.

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Introduction

The heat shock protein subfamily of 90 kDa belongs to a group of highly conserved stress proteins and it is one of the most abundant proteins in eukaryotic cells, comprising 1-2% of the total protein under non-stress conditions [1]. Five isoforms of Hsp90 have been identified that differ in their cellular localization and abundance. In particular, Hsp90 α and Hsp90 β are the major cytosolic isoforms, sharing approximately 85% sequence identity

at the protein level. The structure of both Hsp90 isoforms consists of an N-terminal ATP-binding domain and a C-terminal dimerization domain, a middle domain linker region responsible for substrate-protein interactions, and a following charged domain that promotes the dimerization [2, 3]. The ATP binding site is the major target for Hsp90 inhibitors, but until now, specific inhibitors for Hsp90 α or Hsp90 β have not been developed.

In spite of the molecular similarity of these proteins important differences at the transcriptional and transductional levels have been described, suggesting that Hsp90 α and Hsp90 β might have differential roles in the cellular processes in which they are involved. Hsp90 α transcription is tightly regulated by its promoter region, which contains several heat shock elements (HSEs) that are involved in the inducible gene expression of Hsp90 α . However, differences between Hsp90 α and Hsp90 β are found in the promoter region; for Hsp90 β HSEs are found in the first intron of the gene, and is not regarded as a highly inducible protein [4]. Another important difference is that Hsp90 α easily dimerizes, whereas Hsp90 β is mainly found as a monomer; the regions that facilitate these variations reside at the carboxyl-terminal amino acids of Hsp90 [2]. In this regard, it has been reported that the Hsp90 protein dimer, but not the monomer, stabilizes or activates different proteins known as Hsp90 client proteins in a nucleotide-dependent cycle. The Hsp90 cycle includes the following stages: an open, nucleotide-free form; a closed, ATP-bound structure; and a closed, ADP-bound conformation [5]. The closed conformation had been suggested to form stable client-protein complexes, emphasizing the importance of Hsp90 dimerization for activating the client proteins [6]. Thus, Hsp90 is a crucial regulator of a vast array of cellular processes by interacting with more than 100 client proteins (<http://www.picard.ch/downloads/Hsp90interactors>). Included among these proteins are steroid hormones, transcription factors, kinases, G proteins subunits, and endothelial nitric oxide synthase (eNOS).

eNOS is the primary source of nitric oxide (NO) in endothelial cells. It has been demonstrated that the interaction between Hsp90 and eNOS increases the activity of this enzyme, with greater NO production as a result. In contrast, the dissociation of these complex is not only associated with a reduction of NO synthesis but also turns eNOS into a superoxide generator [7-9]. Recent studies have shown that Hsp90 binds to the N-terminal oxygenase domain of eNOS present in residues 301-320 [9]. This interaction has a physiological connotation, because eNOS activity is regulated by at least four well-

described mechanisms: 1) by enhancing calmodulin binding affinity to eNOS [10, 11]; 2) by facilitating the ability of Ca²⁺/calmodulin to dissociate the interaction between caveolin-1 and eNOS, which reverses the inhibitory action of caveolin-1 on eNOS [12]; 3) by propitiating eNOS serine 1179/1177 (bovine/human) phosphorylation, a translational modification that activates this enzyme [13]; and 4) by recruiting Akt/PKB, which facilitates eNOS phosphorylation in the serine residue 1179 [14]. However, in the eNOS activation by Hsp90, the specific role of the α or β isoforms has not been completely addressed. To explore the specific participation of each Hsp90 isoform, this study was designed to evaluate the effect of Hsp90 α or Hsp90 β over-expression on the NO/eNOS pathway in human embryonic kidney (HEK-293) cells.

Materials and Methods

Plasmid constructs

Hsp90 α and Hsp90 β genes were amplified by PCR from cDNA synthesized from total rat RNA by using the forward primers 5' AAG GAAAAAGC GGC CGC TCG TCAAGATGC CTG AGG AA 3' and 5' AAG GAAAAAGC GGC CGC CTG CCA AGA TGC CTG AGG AA 3', respectively, and the reverse primers 5' CTA GTC TAG AGA GCC TTT AAT CCA CTT CTT CC 3' and 5' CTA GTC TAG ATT CTG GTG AAG CCT AGT CTA CTT C 5', respectively. In the forward primers, a *Not I* restriction site was included in the sequence, whereas in the reverse primers, a *Xba I* restriction site was introduced. The amplified fragments of 2260 for Hsp90 α and 2220 bp for Hsp90 β were cloned into the pcDNA3.1 (+) vector (Invitrogen) by using the *Not I* and *Xba I* restriction sites. The identities of the constructions were confirmed by automatic sequencing.

Hsp90 plasmid isolation

The isolation of the plasmids containing Hsp90 α or Hsp90 β sequences (pc-Hsp90 α or pc-Hsp90 β , respectively) was performed using the same general strategy for the large-scale isolation described by Qiagen Science (Maryland, USA). The DNA was crudely checked for concentration and purity, using 1% agarose gel electrophoresis against molecular weight markers standards (λ DNA/*Hind III* fragments from Invitrogen, Carlsbad, CA), and it was then corroborated with the molecular weights reported for the Hsp90 α or Hsp90 β gene. The DNA plasmid yield was 10-15 μ g in a 10-ml starting culture.

Cell Culture and Transfection

Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 10% fetal bovine serum (FBS) (Life Technology), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C with 5% CO₂. One day before the transfection, 5 x 10⁵ HEK-293 cells were plated in 1 ml of DMEM medium per well in

six-well culture plates until they reached 90% confluence. To determine the amount of plasmid/liposome complex for obtaining the greater Hsp90 α or Hsp90 β over-expression, 0.5–2 μ g of Hsp90 α plasmid (pcDNA3.1/Hsp90 α) or 4–8 μ g of Hsp90 β plasmid (pcDNA3.1/Hsp90 β) was diluted in 100 μ l of Opti-MEM[®] reduced serum medium (Invitrogen, Carlsbad, CA) and incubated for 15 min at room temperature. Next, 4 μ l of Lipofectamine[™] 2000 transfection reagent (Invitrogen, Carlsbad, CA) in 100 μ l of Opti-MEM reduced serum was added, and the samples were maintained at room temperature for an additional 15 min. Thereafter, 200 μ l of each Hsp90 complex was added to each well containing cells. The optimal transfection time for obtaining the higher over-expression of Hsp90 α or Hsp90 β was assessed at 24, 48 and 72 hours after transfection. Thus, the optimal conditions for Hsp90 α and Hsp90 β over-expression in HEK-293 cells were 1 μ g of pc-Hsp90 α or 6 μ g of pc-Hsp90 β and 48 hr after transfection. Cells transfected with the pcDNA3.1(+) empty vector were included as controls in each experiment. All sets of transfections were performed at least in triplicate.

Small-interfering RNA (siRNA) transfection

The silencer pre-designed siRNAs corresponding to the target sequence of Hsp90 α (Cat. number 4390818) or Hsp90 β (Cat. number AM16831) were purchased from Ambion Inc. (Austin, TX). A silencer negative siRNA was used as a negative control (Cat. Number AM4613). In six-well plates, proliferating HEK-293 cells (at 70–90% confluency) were cultured and transfected with 100 nM of Hsp90 α siRNA or Hsp90 β siRNA. After 24 hr of transfection, Western blot analysis was carried out to examine the knockdown of targeted proteins and nitric oxide production.

Western Blot Analysis

Proteins from cell cultures transfected with pcDNA3.1/Hsp90 α , pcDNA3.1/Hsp90 β , pcDNA3.1, siRNA-Hsp90 α , or siRNA-Hsp90 β were extracted using a lysis buffer containing 50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, a protease inhibitor cocktail (Complete, Roche) and phosphatase inhibitors, followed by centrifugation at 14000 rpm. Thereafter, the supernatants containing the proteins were recovered, and samples containing 20 μ g of total protein were resolved by 8.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (PVDF, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were then blocked in 5% blotting solution (Bio-Rad) and incubated with their respective specific antibodies, as detailed below. The lower part of the membranes was incubated with a goat anti-actin antibody (1:5000) overnight at 4°C (Santa Cruz Biotechnology, Santa Barbara, CA), whereas the upper membranes were incubated with polyclonal anti-rabbit Hsp90 β (1:5000, Abcam Inc. Cambridge, MA), polyclonal anti-rabbit Hsp90 α (1:5000, Abcam Inc. Cambridge, MA), anti-rabbit eNOS (1:500, Cell Signalling Technology), polyclonal anti-rabbit phosphorylated eNOS Ser1177 (1:500, Cell Signalling Technology), polyclonal anti-rabbit phospho-eNOS Thr495 (1:500, Cell Signalling Technology), polyclonal anti-human Akt (1:1000, Santa Cruz, Biotechnology), or polyclonal anti-human

phospho-Akt Ser473 (1:1000, Santa Cruz, Biotechnology) antibodies. Membranes were then incubated with the secondary HRP-conjugated rat anti-rabbit IgG antibody (1:5000, Alpha Diagnostics, San Antonio, TX). Proteins were detected with an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Amersham) and autoradiography. The bands were scanned for densitometric analysis.

Analysis of dimer and monomer conformation

The Hsp90 α and Hsp90 β dimer/monomer ratio was evaluated by Western blot for non-denatured proteins. Non-boiled samples containing 10 μ g of total protein were resolved in 7.5% PAGE at 4°C. Proteins were transferred to a polyvinylidene difluoride membrane, and Western blot analysis for Hsp90 α or Hsp90 β was performed as described above. For eNOS dimer/monomer conformation analysis, non-boiled samples containing 50 μ g of total protein were resolved in a 6% SDS-PAGE at 4°C. Then, proteins were transferred to a polyvinylidene difluoride membrane, and Western blot analysis for eNOS was performed as described above.

Immunoprecipitation (IP) analysis

Subconfluent proliferating cells in 300-mm² dishes were harvested, and whole cell extracts were prepared and subjected to IP. After 48 h of transfection, the cells were washed with 2 ml of phosphate buffered saline (PBS), and lysis ELB buffer was added. Cell debris was removed from the cell lysates, and eNOS protein was immunoprecipitated using a protein G immunoprecipitation kit (Sigma Aldrich, Inc. St Louis, MO). Each IP was performed using 3 μ g of eNOS antibody (BD Transduction Laboratories) and 1 mg of cell protein. The immunoprecipitated proteins were eluted by boiling in Laemmli sample buffer, separated by 8.5% SDS-PAGE, transferred onto PVDF membranes and subjected to Western blotting. The membrane was blocked with 5% non-fat milk in TBS-Tween (0.1%) and then immunoblotted for eNOS and Hsp90 α (Abcam Inc., Cambridge, MA), as previously described. Next, the membranes were stripped and reprobed with anti-Hsp90 β (1:5000 dilution, Abcam Inc., Cambridge, MA) and eNOS (1:500 dilution) antibodies to demonstrate equal amounts of eNOS immunoprecipitation in all samples.

Hydrogen Peroxide Determination

The amount of hydrogen peroxide was determined in whole cell lysates (WCL) from each group of cells transfected using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen 29851 Willow Creek Road), according to the instructions provided. Briefly, 50 μ l of each WCL or standard curve (H₂O₂ 1–10 μ M) was mixed with 50 μ l of the Amplex Red reagent/HRP in a microplate. The samples were incubated for 30 minutes protected from light to allow the reaction between H₂O₂ and the Amplex reagent to occur. The plate was read at 560 nm. The amount of H₂O₂ detected in the samples is expressed as nM/ μ g of protein.

Measurement of NO release or NO Assay

NO production was indirectly determined in WCL from each transfected group using the colorimetric Nitric Oxide Assay

Fig. 1. A) Hsp90α protein levels in HEK-293 cells transfected with different amounts of pc-Hsp90α construct (black bars) compared with liposome-treated HEK-293 cells (white bars). Greater over-expression was obtained with 1 μg of pc-Hsp90α, B) without modifying endogenous Hsp90α. C) Western blot analysis of Hsp90α, showing that the higher efficiency of over-expression was obtained after 48 h of pc-Hsp90α transfection, D) without modifying endogenous Hsp90α. E) After 48h of transfection using 1 μg of pc-Hsp90α, Hsp90α protein levels were significantly increased, F) without modifying basal Hsp90β expression. These experiments were performed in at least three different cell transfected cultures. * $p < 0.05$ vs. empty vector-transfected cells.

Kit (Oxford Biomedical Research Inc., Oxford, MI). This assay consists of reducing the sample nitrates to nitrites with the use of nitrate reductase, followed by nitrite quantification with the Griess reaction and detection at 540 nm. The protein concentration in each WCL was determined by the Lowry assay (Bio Rad, Hercules, CA) and in duplicate. The concentration of nitrites in the WCL is expressed as μM/μg of protein.

Superoxide anion detection

Intracellular superoxide detection was assessed by hydroethidine staining (Polysciences, Inc., Warrington, PA). A stock solution of hydroethidine (HE) was prepared by dissolving 7 mg of HE in 1 mL of N,N-dimethylacetamide. The working solution was prepared by the addition of 20 μl of HE to 10 ml of PBS, and this mixture was filtered through a 0.22-μm Millipore filter. After 48 h of transfection, the medium was removed, and the cells were washed three times with PBS. The staining solution was added, and the cultures were incubated for 15 minutes at room temperature and protected from the light. Thereafter, the staining solution was removed, and the cells were washed three times. The cells were re-suspended in PBS for flow cytometry analysis. Ten thousand cells from each transfection group were analyzed in a FACScan cytometer

(Becton Dickinson), and the fluorescence intensity was determined using an excitation of 535 nm and an emission of 610 nm. The data were analyzed using the Cell Quest program.

Statistical analysis

Results are presented as means ± SE. The significance of the differences among groups was tested by ANOVA using Bonferroni's correction for multiple comparisons and between two groups by the un-paired T test. Statistical significance was defined as a p value < 0.05 .

Results

Hsp90α cloning and HEK-293 transfection

The Hsp90α gene was amplified from rat kidney cDNA using specific primers, as detailed above. After the proper purification of PCR product of ≈ 2260 bp, rat Hsp90α (rHsp90α) was cloned into the pcDNA3.1(+) expression vector via a multi-cloning site by using *Xba I* and *Not I* sites. After *Xba I* and *Not I* digestion, two

Fig. 2. A) Hsp90 β protein levels in HEK-293 cells transfected with different amounts of pc-Hsp90 β construct (black bars) compared with liposome-treated HEK-293 cells (white bars). The greater over-expression was obtained with 6 μ g of pc-Hsp90 β . B) Western blot analysis of Hsp90 β showing that the higher efficiency of over-expression was obtained after 48 h of pc-Hsp90 β transfection. C) After 48h of transfection using 6 μ g of pc-Hsp90 β , Hsp90 β protein levels were significantly increased, D) without modifying basal Hsp90 α expression. These results were obtained from at least two different experiments * $p < 0.05$ vs. empty vector-transfected cells.

fragments were obtained, with the larger corresponding to the pcDNA3.1 vector and the smaller to the Hsp90 α sequence (data not shown). The sequence of Hsp90 α was corroborated by automatic sequencing that revealed 99% identity with the rat sequence reported for Hsp90 α in GenBank (number AJ428213.1). Similarly, Hsp90 α was cloned in the expression vector pcDNA3.1/NT-GFP (which codifies for the green fluorescent protein). Epifluorescence inverted microscopy was employed to detect the transfection efficiency. It was shown that 80% of HEK-293 cells were able to express the GFP (data not shown).

Different concentrations of Hsp90 α plasmid were transfected into HEK-293 cells to determine the optimal conditions for pcDNA 3.1/Hsp90 α transfection. The upper panel of Fig. 1A shows the insets of the Western blot analysis for Hsp90 α and β actin. Two bands in the Hsp90 α Western blot were detected. The larger band corresponds to the human endogenous Hsp90 α that is expressed in HEK-293 cells, and the lower band corresponds to the transfected rat Hsp90 α . Greater rat Hsp90 α over-expression was detected when the cells were transfected with 1 μ g of pcDNA 3.1/Hsp90 α without changes in the endogenous Hsp90 α expression, as shown in Fig. 1B. In addition, different times after transfection were assessed to determine the greatest Hsp90 α over-expression. As shown in Fig. 1C, the higher rat Hsp90 α expression was obtained after 48 h, without changes in human Hsp90 α expression (Fig. 1D). Other groups of cells were transfected with the optimal conditions, then Hsp90 α and Hsp90 β protein levels were detected by

Fig. 3. Western blot analysis showing basal protein levels of eNOS, Hsp90 α , Hsp90 β and β -actin in HEK-293 intact cells.

Western blot analysis. As expected, pcDNA3.1/Hsp90 α transfection increased Hsp90 α protein levels, as shown in Fig. 1E, without altering Hsp90 β protein levels as is represented in Fig. 1F. These results shown that Hsp90 α and Hsp90 β antibodies were specific to detect each Hsp90 isoform studied.

Hsp90 β cloning and HEK-293 transfection

The Hsp90 β gene was also amplified from rat kidney cDNA using specific primers, as detailed above. The PCR product of ≈ 2220 bp was purified and cloned into pcDNA3.1(+) using *Not I* and *Xba I* restriction sites. The Hsp90 β sequence was first corroborated by restriction analysis; for this, the vector was digested with *Xba I* and *Not I*, *Xba I*, *Sac I*, *EcoR I*, *Kpn I*, and *Mfe I*. When the vector was digested with *Xba I* and *Not I*, two fragments were obtained, with the larger corresponding

Fig. 4. Effects of pc-Hsp90α and pc-Hsp90β transfection on eNOS/NO pathway. A) Change in percentages of NO₂/NO₃ levels in the lysates of HEK-293 cells transfected with pc-Hsp90α (gray bars), pc-Hsp90β (black bars), siRNAHsp90α (white bars), and siRNA Hsp90β (dark gray bars). B) Co-immunoprecipitation of eNOS and Hsp90α or Hsp90β from HEK-293 cell lysates transfected with either Hsp90α or Hsp90β respectively. C) eNOS expression detected by Western blot analysis from HEK-293 cells transfected with empty vector, Hsp90α or Hsp90β. D) eNOS Ser1177 phosphorylation, E) eNOS Thr495 phosphorylation, and F) Akt expression and phosphorylation at residue Ser437, determined by Western blot analysis using specific phospho-antibodies in the studied groups of HEK-293 cells. The proteins were isolated from at least three different cell transfected cultures *p<0.05 vs. liposome-transfected cells. **p<0.05 vs. pc-Hsp90α or pc-Hsp90β-transfected cells.

to the pcDNA3.1 vector and the smaller to Hsp90β (data not shown). With the rest of the enzymes, we obtained the expected specific size fragments for Hsp90β (these size fragments are not obtained when Hsp90α was digested with these enzymes). The sequence was also corroborated by automatic sequencing, which revealed 100% identity with the rat sequence reported for Hsp90β in GenBank (number DQ022068.2). To determine the optimal conditions for Hsp90β over-expression, HEK-293 cells were transfected with different plasmid concentrations. As shown in Fig. 2A, maximum over-expression was detected when cells were transfected with 6 μg of pcDNA 3.1/Hsp90β a unique band of Hsp90β was detected corresponding to both human endogenous and rat transfected sequence. In addition, the best period of time to obtain maximum over-expression with 6 μg of plasmid was at 48 h after transfection (Fig. 2B). Independent groups of cells were transfected with the optimal conditions, and Hsp90α and Hsp90β protein levels

were detected by Western blot analysis. As expected, pcDNA3.1/Hsp90β transfection increased Hsp90β by ≈2-fold, as shown in Fig. 2C, without modifying the Hsp90α protein levels (Fig. 2D).

Effects of Hsp90α and Hsp90β over-expression on the eNOS/NO pathway

Because it has been shown that Hsp90 regulates eNOS activity [10-13, 15], we studied the specific role of Hsp90α and Hsp90β over-expression in nitric oxide production. First we assessed eNOS protein levels in untransfected HEK-293, as Fig. 3 shows these cells do express eNOS, as well as Hsp90α and Hsp90β. This finding was further confirmed by the measurement of NO₂/NO₃ levels in these cells. Figure 4A shows the percentage changes in NO₂/NO₃ levels in groups transfected with Hsp90α or Hsp90β, compared with the cells that were transfected with the empty pcDNA vector. Hsp90α over-expression produced a significant increase

Fig. 5. Effects of Hsp90 α and Hsp90 β over-expression on oxidative stress in HEK-293 cells. A) Hydrogen peroxide levels in lysates of HEK-293 cells transfected with empty vector (white bars), pc-Hsp90 α (dark gray bars), and pc-Hsp90 β (black bars). Hydroethidine staining was assessed as a measure of superoxide anion production and was quantified by flow cytometry. B) Upper panel, representative histograms of the three groups of transfected HEK-293 cells, showing a greater hydroethidine staining in the group that was transfected with pc-Hsp90 β . C) The percentage of HEK-293 cells positive for hydroethidine staining and D) Fold increase in the Mean Fluorescence Intensity (FMI). These results were performed with at least three different transfected cultures. * $p < 0.05$ vs. empty vector-transfected cells.

in NO metabolites (from 0.29 ± 0.01 to 0.61 ± 0.03 $\mu\text{M}/\mu\text{g}$ of protein) that represented an increase of 113.3%. Intriguingly, Hsp90 β over-expression produced the opposite effect, such that NO₂/NO₃ levels were significantly reduced in transfected HEK-293 cells (0.13 ± 0.01 $\mu\text{M}/\mu\text{g}$ of protein). The specificity of the observed effects was studied by reducing the endogenous Hsp90 α and Hsp90 β expression by using specific siRNAs for these proteins. Therefore, other groups of cells were transfected with the corresponding siRNA/Hsp90 α or siRNA/Hsp90 β . We assessed the Hsp90 α and Hsp90 β expression after 24 to 72 h after respective siRNAs transfection and from these results the experiments with siRNAs were performed after 48h (data not shown). When the NO₂/NO₃ levels were evaluated, we observed that siRNA/Hsp90 α transfection produced a significant reduction in NO metabolites, by 36.5 % as is depicted in Fig. 4A. In contrast, siRNA/Hsp90 β transfection was associated with a significant increase in NO₂/NO₃ levels, by 74.7%. These data strongly suggest that Hsp90 α and Hsp90 β have differential roles in regulating eNOS activity.

We examined whether these differences were propagated by changes in the interaction of Hsp90 α or Hsp90 β with eNOS. As shown in Fig. 4B, it was not the case, as the IP-Western blotting analysis revealed that eNOS was able to associate with both Hsp90 α and Hsp90 β , suggesting that the reduction of eNOS activity by Hsp90 β did not result from an inability of this chaperone to associate with eNOS.

To evaluate if the differential modification of NO production induced by Hsp90 α and Hsp90 β over-expression was mediated by changes in eNOS expression and its phosphorylation, eNOS protein levels and the phosphorylation of serine 1177 and threonine 497 residues of eNOS were evaluated in transfected HEK-293 cells by Western blot analysis using the corresponding phospho-antibodies. The eNOS protein levels in HEK-293 cell cultures was not modified by either Hsp90 α or Hsp90 β transfection, as evident in the top inset and by the densitometric analysis shown in Fig. 4C. A significant increase in the immune-detection signal of phosphorylated Ser1177 was observed in the cells that over-expressed

Fig. 6. Effects of Hsp90α or Hsp90β over-expression on the monomer and dimer conformations. A) Hsp90α Western blot analysis of non-denatured proteins extracted from HEK-293 cells transfected with empty vector (white bars) pc-Hsp90α (dark gray bars), and pc-Hsp90β (black bars). The upper panel shows a representative image of the Hsp90α Western blot, and the lower panel shows graphical representations of the dimers, monomers and dimer/monomer ratios of Hsp90α. B) Hsp90β Western blot analysis of non-denatured proteins extracted from HEK-293 cells transfected with empty vector (white bars), pc-Hsp90α (dark gray bars), and pc-Hsp90β (black bars). The proteins were extracted from three different HEK-293 transfected cell cultures. *p<0.05 versus empty transfected cells.

Fig. 7. Effect of Hsp90α and Hsp90β over-expression on eNOS dimer/monomer conformation. A) A representative image of eNOS Western blot from total proteins extracted from HEK-293 cells transfected with empty vector (open bars), Hsp90β (black bars), and Hsp90α (gray bars), respectively. Two bands were detected: 280kDa and 140kDa, corresponding to eNOS dimeric and monomeric conformations, respectively. B and C) dimeric and monomeric eNOS densitometric analysis, and D) eNOS dimer/monomer ratio (D/M). The results reported here were repeated two times with similar results. *p<0.05 vs. empty vector transfected cells.

Hsp90α, an effect that was not seen in Hsp90β-transfected cells (Fig. 4D). The mean ratio of phosphorylated Ser1177 eNOS/β-actin in pc-Hsp90α-transfected cells was 0.83 ± 0.08 , compared to 0.32 ± 0.04 and 0.36 ± 0.05 AU for pc-Hsp90β and pcDNA3.1 empty vector, respectively. In contrast, the transfection of pc-Hsp90β significantly increased

Thr495 eNOS phosphorylation (Fig. 4E), but over-expression of Hsp90α did not modify the phosphorylation of this eNOS residue. The mean ratio of phosphorylated Thr495-eNOS/β-actin in Hsp90β and pcDNA3.1-transfected cells was 0.86 ± 0.1 , compared to 0.50 ± 0.05 and 0.55 ± 0.09 in liposomes and Hsp90α-transfected cells, respectively.

Because it has been reported that Hsp90 plays an important role in the activation of p-Akt/PKB [16] and because this kinase is able to phosphorylate Ser1177 of eNOS [17, 18], we evaluated the effect of over-expressing Hsp90 α or Hsp90 β on Akt/PKB activation. As shown in Figure 4F, the transfection of either Hsp90 α or Hsp90 β did not modify the Akt/PKB protein levels, but a different effect was observed for the phosphorylation of this kinase. Thus, Hsp90 α over-expression was associated with a significant increase in Akt/PKB phosphorylation, whereas Hsp90 β over-expression induced a significant reduction in the amount of phosphorylated Akt/PKB.

Moreover, it has been previously shown that eNOS is able to generate superoxide anion when it is phosphorylated at Thr-497; thus, we assessed the effect of Hsp90 α or Hsp90 β over-expression on the cellular oxidative stress state by measuring the hydrogen peroxide levels in HEK-293 cell cultures. Figure 5A shows the H₂O₂ levels in WCL, expressed as nmol/ μ g of protein. It is noteworthy that the H₂O₂ levels increased by two-fold when Hsp90 β was over-expressed, an effect that was not observed when pc-Hsp90 α was transfected. To further confirm these findings, we performed hydroethidine (HE) staining to detect intracellular superoxide anion generation by flow cytometry analysis. Figure 5B shows the representative histograms of each transfected group, and Figure 5C and 5D show the percentage of stained cells and the quantification of the mean fluorescence intensity (MFI), respectively. The HE staining confirmed that Hsp90 β over-expression induced a higher generation of superoxide anion, as shown by the increase in the percentage of cells positive for HE staining (from 2.7 ± 0.9 to 9.0 ± 0.8 %, $p=0.002$) and by the MFI measurement (from 1 ± 0.06 to 1.6 ± 0.03 AU, $p=0.002$).

Because the dimeric Hsp90 conformation has been shown to be necessary for interactions with the client proteins, the monomeric/dimeric ratio in HEK-293 cells transfected with Hsp90 α and Hsp90 β was evaluated. The insets in Fig. 6A and Fig. 6B show Westerns blot under non-denaturing conditions of proteins extracted from Hsp90 α and Hsp90 β -transfected cells, respectively. Typically, most of the Hsp90 α was found in its dimeric conformation (Fig. 6A), in contrast with the Hsp90 β which was found mainly in the monomeric conformation (cells transfected with the empty vector) as is shown in Fig. 6B. When the monomeric/dimeric ratio was evaluated in cells transfected with either pc-Hsp90 α or pc-Hsp90 β most of the Hsp90 α was found in its dimeric conformation. In addition, a greater amount of this protein

was detected in the cells that were transfected with pc-Hsp90 α . In contrast, when Hsp90 β was over-expressed, most of the protein was found in its monomeric conformation, and again, more monomeric Hsp90 was observed in the cells that were transfected with pc-Hsp90 β (Fig. 6B).

It has been previously reported that dimeric eNOS conformation is required for a suitable NO production [19], thus we evaluated if Hsp90 α and Hsp90 β over-expression modify the dimeric/monomeric eNOS conformation. The upper panel of Figure 7 shows the inset of eNOS Western blot analysis in non-denaturing conditions from HEK-293 cells transfected with empty vector, pcHsp90 α and pcHsp90 β respectively. The lower panel depicts the densitometric analysis of dimeric and monomeric bands of eNOS, together with the resulting dimeric/monomeric ratio. The reduction of NO synthesis induced by Hsp90 β over-expression was associated with a significant decrease in eNOS dimeric conformation, with the concomitant eNOS dimeric/monomeric ratio reduction. This effect was not seen in pcHsp90 α transfected cells.

Discussion

Hsp90 plays a key role in cellular signal transduction networks, as it is essential for maintaining the activity of numerous signaling proteins. One of the most studied Hsp90 interactions is that established with eNOS. Several studies have shown that Hsp90 regulates eNOS activity through modulations of the following processes: helping to activate eNOS by dissociating it from caveolin-1; enhancing eNOS enzymatic activity by promoting its dimerization; facilitating the activating eNOS phosphorylation; and modulating the release of NO vs. superoxide by eNOS [10-13]. Furthermore, Hsp90 has also been proposed to facilitate the calcineurin-dependent dephosphorylation of the residue Thr495 site of eNOS, which contributes to enhance its enzymatic activity [15]. Therefore, Hsp90 plays a critical role in vascular NO synthesis through the regulation of eNOS activity. In this regard, we have recently shown that Hsp90 inhibition with radicicol, that reduced Hsp90 interaction with their client proteins, produced a significant reduction in renal plasma flow and glomerular filtration rate, an effect that was associated with a decreased urinary excretion of nitrites and nitrates (NO₂/NO₃), suggesting that the disruption of eNOS-Hsp90 complex reduced renal NO synthesis [20]. In addition, previous studies have shown that the disruption of eNOS/Hsp90 complex promotes not only the reduction

of NO but also the generation of O_2^- anion by eNOS [8, 9, 21, 22]. Even though there is enough evidence about the Hsp90/eNOS coupling and uncoupling in NO and O_2^- generation, respectively, the specific role of Hsp90 α and Hsp90 β in this pathway have not been completely addressed. Two previous studies [7, 23] suggested that transfection of Hsp90 β induces an increase in NO production in COS and pulmonary arterial endothelial cells (PAEC). However, results from these studies are hard to evaluate because the Hsp90 antibody that was used recognizes both isoforms and thus Hsp90 β over-expression was not demonstrated. In addition, in one of the studies [23], the effect of empty adenovirus transfection was not evaluated. Other studies in which the interaction between eNOS and Hsp90 was enhanced by a transient Hsp90 transfection, did not specify which isoform of Hsp90 was used for the transfection [15, 24].

In the present study, the participation of Hsp90 α or Hsp90 β in the eNOS/NO/ O_2^- pathway was evaluated by inducing the over-expression of each isoform in HEK-293 cells. For this issue, we cloned Hsp90 α and Hsp90 β into an expression vector and the over-expression was gotten by transitory transfection. The pc-Hsp90 α transfection produced a significant elevation in Hsp90 α without modifying Hsp90 β protein levels. Similarly, in cells transfected with pc-Hsp90 β , the levels of this protein were significantly enhanced, whereas the endogenous Hsp90 α protein levels were not affected. Therefore, the effects seen in this study on the NO/eNOS pathway were solely induced by the transitory over-expression of each Hsp90 isoform.

Accordingly, we observed that Hsp90 α over-expression was associated with a significant increase in NO_2/NO_3 levels. Quite interestingly, pc-Hsp90 β transfection produced the opposite effect; thus, NO metabolites were significantly reduced in HEK-293 cells. These findings were supported by results using respective siRNAs of Hsp90 α or Hsp90 β . Consequently, when Hsp90 α expression was reduced by this strategy, the NO metabolites were significantly decreased. In contrast, when HEK-293 cells were transfected with Hsp90 β siRNA, the NO_2/NO_3 levels were significantly enhanced. In this regard, these results suggest that cytosolic Hsp90 isoforms exert distinctive functions in the NO pathway, in which Hsp90 α positively regulates NO synthesis whereas Hsp90 β has a contrary effect.

To begin to understand the mechanisms by which Hsp90 α and Hsp90 β have differential roles in NO production, we evaluated the eNOS protein levels and the eNOS phosphorylation of two main residues that are

implicated in this enzyme activity. We found that eNOS protein levels were not modified when the HEK-293 cells were transfected with either Hsp90 α or Hsp90 β , however, profound changes in its phosphorylation state were observed. Hsp90 α over-expression was associated with a significant increase in eNOS-activating phosphorylation (Ser1177), whereas Hsp90 β over-expression produced an increase in the eNOS-inactivating phosphorylation (Thr495). In this regard, previous studies have shown that increased eNOS phosphorylation at Thr495, as caused by eNOS-Hsp90 uncoupling, resulted in lesser NO production [20, 25, 26]. In support of this, Lin et al. [27] reported that the T495A eNOS mutant, which cannot be phosphorylated at this site, was able to enhance eNOS activity. The immunoprecipitation analysis performed in this study revealed that both Hsp90 α and Hsp90 β are able to associate with eNOS, suggesting that these two Hsp90 isoforms exert differential eNOS regulation through modulating eNOS phosphorylation. Incidentally, it has been shown that eNOS activation by Hsp90 is mediated, in part, by a scaffolding effect that enhances the interaction between eNOS and the active serine/threonine kinase Akt/PKB [10], which, in turn, promotes eNOS Ser1177 phosphorylation. Intriguingly, Akt/PKB activity is also regulated by Hsp90 through the prevention of PP2A-mediated Akt dephosphorylation [16]. Consequently with these previous findings, we observed that pc-Hsp90 α transfection increased Akt/PKB phosphorylation, suggesting that the greater NO synthesis observed in these transfected cells was in part due to an enhanced Akt/PKB activity, resulting in eNOS Ser1177 phosphorylation. Of note, pc-Hsp90 β transfection induced a significant reduction of Akt/PKB phosphorylation, which was associated with an increase in the inactivating eNOS phosphorylation (Thr495), and these findings were associated with a significant reduction in NO production. Thus, our results suggest that another way by which Hsp90 α and Hsp90 β differentially regulate eNOS is by modifying Akt/PKB phosphorylation.

Several studies have shown that eNOS Thr495 phosphorylation promotes the superoxide anion (O_2^-) generation instead of NO [8, 9, 23, 28, 29]. To explore the significance of the increased eNOS Thr495 phosphorylation induced by pc-Hsp90 β transfection on O_2^- generation, the levels of peroxide hydrogen and O_2^- anion were evaluated in transfected HEK-293 cells. The reduction of NO induced by Hsp90 β over-expression was associated with a significant increase in hydrogen peroxide, suggesting a greater O_2^- generation. These results were confirmed by measuring cellular O_2^- by

cytofluorometry, in which a significant increase in the generation of this anion was observed in HEK-293 cells transfected with pc-Hsp90 β , an effect that was not seen following transfection with pc-Hsp90 α . Altogether, these results suggest that Thr 497 phosphorylation switches the ability of eNOS to produce O₂⁻, and, interestingly, this effect seems to be promoted by the association of Hsp90 β with eNOS.

The dimeric molecular conformation of Hsp90 is required for the activation and stabilization its client proteins. Hsp90 consists of a C-terminal dimerization domain, a middle domain (which may interact with substrate proteins), and an N-terminal ATP-binding domain. The activation process depends on the hydrolysis of ATP by Hsp90. Thus, the ability of Hsp90 to interact with its client proteins is strongly determined by its conformational state [3, 30, 31]. In this sense, Hsp90 β human and mouse is less frequently found as a dimer than Hsp90 α . The impeded ability of Hsp90 β to dimerize is related to the 16 amino acid substitutions located in the 561-685 amino acid region of the C-terminal dimerization domain [32]; specifically, Thr566 and Ala629 seem to be responsible for the reduced ability of Hsp90 β to dimerize [33]. Here, we evaluated the monomer/dimer ratio of Hsp90 α and Hsp90 β in HEK-293 cells before and after pc-Hsp90 α or pc-Hsp90 β transfection. As has been previously reported, we found that Hsp90 α was predominantly in its dimeric molecular conformation, whereas Hsp90 β persisted in a monomeric conformation in the cells that were transfected with the empty vector. When Hsp90 α was over-expressed, most of the protein was found in the dimeric form, without changes in the Hsp90 β monomer form. In contrast, the over-expression of Hsp90 β rendered a greater proportion of the monomeric conformation. These results suggest that both Hsp90 isoforms are able to associate with eNOS, but the conformation state of Hsp90 seems to be a regulator of eNOS ability to produce NO or O₂⁻. Because Hsp90 is also able to interact with neuronal and inducible NOS [34-36], we can not exclude the participation of other NOS isoforms in the NO production seen when Hsp90 α or Hsp90 β were over-expressed.

The differential regulation of eNOS by Hsp90 α and Hsp90 β seems not only to be explained by changes in eNOS phosphorylation or Hsp90 conformation, but also, Hsp90 β over-expression induced a significant reduction in eNOS active conformation. Thus, the reduction in NO synthesis observed in HEK-293 cells transfected with pcHsp90 β was associated with a lesser dimeric conformation of eNOS, suggesting that this is another mechanism by which Hsp90 β differentially regulates eNOS activity.

Taken together, our results suggest that to obtain a suitable NO synthesis, the formation of the dimeric conformation of Hsp90 α is required, which not only allows eNOS dimerization, but also helps to eNOS phosphorylation at Ser1177 by Akt/PKB. In contrast, when Hsp90 β is predominantly found as a monomer, it affects eNOS pathway through two different mechanisms: by reducing Akt/PKB phosphorylation/activity and by propitiating the inactive eNOS conformation which in turns is associated with eNOS phosphorylation at Thr495, an effect that causes eNOS to produce O₂⁻ instead of NO. These findings could have important implications for abnormal vascular physiology in which the generation of the superoxide anion is increased and nitric oxide availability is reduced.

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