Heat-shock protein 90 augments neuronal nitric oxide synthase activity by enhancing Ca^{2+}/calmodulin binding

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Heat-shock protein 90 (hsp90) has been shown to facilitate neuronal NO synthase (nNOS, type 1) activity *in vivo*. But the direct effect of hsp90 on purified nNOS has not been determined yet. Moreover, the mechanism underlying the action of hsp90 is not known. nNOS activity is primarily initiated and regulated by the binding of Ca^{2+}/calmodulin (CaM). Therefore, we explored whether hsp90 modulates nNOS activity by affecting CaM binding. Recombinant rat nNOS was purified from the stably transfected cells by affinity chromatography. hsp90 increased nNOS activity in a dose-dependent manner with an EC_{50} of 24.1 ± 6.4 nM. In the presence of hsp90, the CaM–nNOS dose–response curve was shifted markedly to the left and the maximal activity was also elevated. Further *in vitro* protein-binding experiments confirmed that hsp90 increased the binding of CaM to nNOS. Taken together, these data indicate that hsp90 directly augments nNOS catalytic function and that this effect is, at least partially, mediated by CaM-binding enhancement.

Key words: calmodulin binding affinity, CaM, hsp90 modulation, nitric oxide synthase catalysis, NOS.

INTRODUCTION

NO is a fundamental signalling molecule participating in a variety of biological processes [1]. Like many other signalling molecules, the production of NO in cells and tissues needs to be modulated precisely because too much or too little NO formation will perturb homoeostasis, leading to disease. In biological systems, NO is produced primarily by a family of NO synthases (NOSs) [2]. Modulation of NO production in cells is therefore achieved mainly by regulation of NOS activity. To date, three NOS isoforms have been identified; neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II) and endothelial NOS (eNOS, type III). Although derived from different genes, the three NOS isoforms exhibit considerable similarity in their structure and function [3,4]. They all utilize L-arginine, oxygen and NADPH as co-substrates to synthesize NO and L-citrulline. The Ca^{2+}/calmodulin (CaM) complex is the common key cofactor that triggers NOS activation. Though most of the agonists, such as glutamate or acetylcholine, initiate nNOS or eNOS activity by raising intracellular Ca^{2+} concentrations, activation of enzymic activity is ultimately determined by the binding affinity of CaM to NOS. For example, because of the high binding affinity between iNOS and CaM, iNOS is fully active under basal Ca^{2+} levels in quiescent cells [2]. Thus modulation of CaM binding profoundly influences NOS activation, and this may serve as another regulatory mechanism to control NO generation from nNOS or eNOS.

Beyond CaM, nNOS and eNOS are also regulated by various scaffolding proteins through protein–protein interactions [5]. For example, nNOS was found to target the membrane by interacting with post-synaptic density proteins PSD-95 and PSD-93 in neurons [6]. nNOS also binds with a 10 kDa protein, PIN, and this binding causes enzymic inhibition [7]. In endothelial cells, eNOS is localized in caveolae through interaction with caveolin-1 [8]. This interaction results in an inhibition of eNOS activity and a decrease in NO production. Recently, heat-shock protein 90 (hsp90), a molecular chaperone, was reported to associate with eNOS and acted as an allosteric enhancer [9]. Subsequent study suggested that hsp90 also facilitated nNOS activation in nNOS-transfected cells [10]. However, this study was conducted in cultured cells and the conclusion about the role of hsp90 in nNOS regulation was largely based on the effects of geldanamycin used as a specific hsp90 inhibitor. Unfortunately, geldanamycin is also known to cause other effects such as tyrosine kinase inhibition and suppression of oncogene expression [11]. Therefore, it remains uncertain whether and how hsp90 directly affects nNOS activity, since the effect of hsp90 on purified nNOS has not been determined. More importantly, the exact mechanism underlying the action of hsp90 is not yet established.

In the present study, we determined directly the effect of hsp90 on nNOS activity using purified nNOS and explored whether hsp90 affects CaM binding to nNOS. Our results from both enzymic measurement and protein-binding assay suggested that hsp90 enhances nNOS activity by facilitating CaM binding.

EXPERIMENTAL

Materials

Cell-culture materials were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). Bovine hsp90 and CaM, both $> 95\%$ pure, were purchased from Sigma (St. Louis, MO, U.S.A.). 2′,5′-ADP–Sepharose 4B was the product of Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). L-[^{14}C]Arginine was purchased from DuPont/NEN (Boston, MA, U.S.A.). NADPH, L-arginine, tetrahydrobiopterin (BH_4), EDTA, *N*-nitro-L-arginine methyl ester (L-NAME) and other reagents were purchased from Sigma unless otherwise indicated.

Abbreviations used: hsp90, heat-shock protein 90; NOS, NO synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; CaM, calmodulin; L-NAME, *N*-nitro-L-arginine methyl ester; BH_4, tetrahydrobiopterin.

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Preparations of recombinant nNOS

Recombinant rat nNOS was purified from stably transfected HEK 293 cells (from Dr Solomon H. Snyder at Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) [12]. The purification protocol was modified slightly from the procedure described in [13]. In brief, nNOS-transfected cells were grown in minimal essential medium with 10% heat-inactivated fetal calf serum. Cells were harvested and homogenized by sonication in buffer A (50 mM Tris/HCl, pH 7.4, 2 mM EDTA, 1 mM PMSF and 10 mM β-mercaptoethanol). After centrifugation (16000 ***g***, 10 min) at 4 °C, the supernatant was applied to a 2′,5′-ADP–Sepharose 4B column pre-equilibrated with buffer A. The column was washed twice with 10 ml of buffer A containing 450 mM NaCl, followed by two washings with 10 ml of Tris/HCl buffer (50 mM, pH 7.4). Then the protein was eluted with 10 mM NADPH in 50 mM Tris/HCl (pH 7.4). The eluate was washed and concentrated using Centricon-100 (Amicon) concentrators. Protein content of the preparations was assayed with Bradford reagent (Bio-Rad) using BSA as standard. The purity of nNOS was determined by SDS/PAGE and visualized with Coomassie Brilliant Blue staining. nNOS activity was assayed by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as described below. Purified nNOS samples were stored in 50 mM Tris/HCl (pH 7.4) buffer with 10% glycerol at −80 °C.

Determination of nNOS activity

nNOS activity was measured by L-[^{14}C]arginine-to-L-[^{14}C]-citrulline conversion assay [14,15]. The reactions were carried out in a total volume of 200 μl of buffer containing 50 mM Tris/HCl, pH 7.4, 3 μM L-[^{14}C]arginine, 0.5 mM NADPH, 1 mM Ca^{2+}, 1 μM BH_4, 10 nM nNOS and different concentrations of hsp90 or CaM. To determine the effects of hsp90, hsp90 was pre-incubated with nNOS for 15 min at room temperature (23 °C). The reaction was initiated by adding nNOS and terminated after 10 min of incubation at 37 °C. L-[^{14}C]Citrulline was separated by passing the reaction mixture through Dowex AG 50W-X8 (Na^+ form, Sigma) cation-exchange columns and quantified by liquid scintillation counting.

In vitro CaM-binding assay

The binding of CaM to nNOS was conducted in 700 μl of binding buffer containing 50 mM Tris/HCl (pH 7.4), 125 mM NaCl, 1 mM Ca^{2+}, 100 μM L-arginine, 1 μM BH_4, 10 nM nNOS and 10 nM CaM in the presence and absence of hsp90 (0.5 μM). After 1 h of incubation at 4 °C, 2′,5′-ADP–Sepharose 4B resins (100 μl of 50% slurry) were added and the mixtures were shaken for 1 h at 4 °C. After binding, the resins were washed extensively six times with high-salt buffer (50 mM Tris/HCl, pH 7.4/450 mM NaCl) followed by one wash with 50 mM Tris/HCl (pH 7.4). The bound proteins were eluted by boiling the resins in SDS sample buffer and then subjected to SDS/PAGE. CaM and nNOS was identified by Western blotting using anti-CaM monoclonal antibody (Upstate Biotechnology) and anti-nNOS monoclonal antibody (Transduction Laboratory).

Statistics

Data are expressed as means ± S.E.M. Comparisons were made using a two-tailed Student's paired or unpaired *t* test. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

We first determined the effect of hsp90 on nNOS activity using purified nNOS preparations. nNOS was isolated from the stably transfected cells by affinity chromatograph. As shown in Figure 1(A), purified nNOS preparations exhibited one prominent band (> 90%) on SDS/PAGE with a molecular mass of 160 kDa, which is consistent with the molecular mass of native nNOS reported previously [3]. Purified nNOS preparations possessed strong catalytic activity, as determined by L-[^{14}C]arginine-to-L-[^{14}C]citrulline conversion assay (Figure 1B). This enzymic activity was blocked by the specific NOS inhibitor L-NAME (1 mM), confirming it was derived from nNOS. NOS activity also required the addition of Ca^{2+}/CaM, a characteristic feature of constitutive nNOS.

To study the effect of hsp90 on nNOS, hsp90 and nNOS were pre-incubated and then nNOS activity was measured. As shown in Figure 2(A), hsp90 dose-dependently increased nNOS activity with an EC_{50} of 24.1 ± 6.4 nM. This was a specific effect by hsp90 because neither heat-denatured hsp90 nor the irrelevant BSA affected nNOS activity (Figure 2B). These data demonstrated that hsp90 specifically enhanced the catalytic function of nNOS.

To assess whether the potentiating effect of hsp90 on nNOS activity involved altered CaM-binding affinity, the dose–response relationship between CaM and nNOS was determined. In the presence of saturated Ca^{2+} (1 mM), CaM increased nNOS activity in a dose-dependent manner (Figure 3). The EC_{50} of CaM for nNOS was 10.2 ± 2.5 nM, which was in agreement with the previously reported value [3]. In the presence of a low level of hsp90 (24 nM), the CaM–nNOS curve was slightly shifted to the left with an EC_{50} value of 6.7 ± 1.2 nM ($P > 0.05$ versus the control). In the presence of 0.5 μM hsp90, the CaM–nNOS response curve was markedly shifted to the left with an EC_{50} value of 1.1 ± 0.23 nM ($P < 0.01$ versus the EC_{50} in the absence of hsp90). The dramatically reduced EC_{50} of CaM for nNOS strongly suggested that the binding affinity between these two proteins was enhanced by hsp90. Interestingly, the maximal activity of nNOS was also significantly increased after hsp90

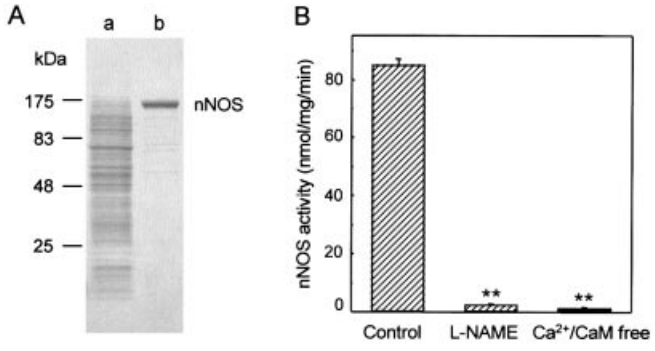

Figure 1 Profile of rat nNOS purified from the stably transfected HEK 293 cells

(**A**) SDS/PAGE of isolated nNOS preparations. Lane a, cell homogenates before purification; lane b, purified nNOS eluted from affinity columns. Proteins were separated on 4–20% gradient gels and visualized by Coomassie Brilliant Blue staining. (**B**) Catalytic activity of purified nNOS preparations. NOS activity was measured by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. The reactions were performed in 200 μl of buffer containing 50 mM Tris/HCl, pH 7.4, 3 μM L-[^{14}C]arginine, 0.5 mM NADPH, 1 mM Ca^{2+}, 588 nM CaM, 1 μM BH_4 and 10 nM nNOS. The preparations showed typical constitutive nNOS characteristics with L-NAME (1 mM)-inhibitory and Ca^{2+}/CaM-dependent activity (**$P < 0.001$, compared with control, $n = 6$).

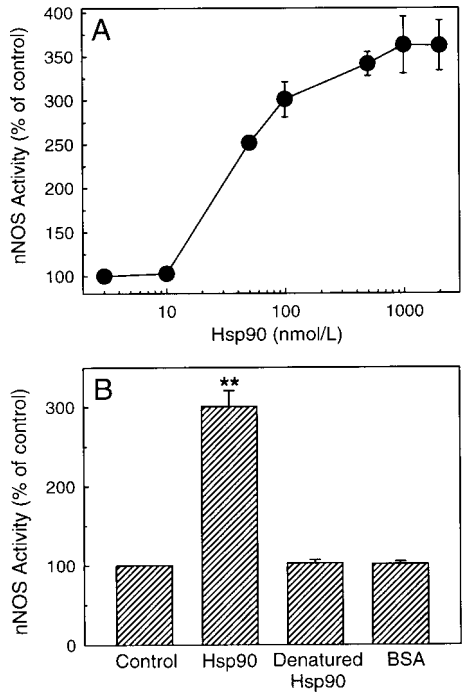

Figure 2 Effect of hsp90 on nNOS activity

(**A**) nNOS activity was measured in the reaction systems consisting of 50 mM Tris/HCl (pH 7.4), 3 μM L-[^{14}C]arginine, 0.5 mM NADPH, 1 mM Ca^{2+}, 12 nM CaM, 1 μM BH_4 and 10 nM nNOS in the absence and presence of hsp90 (1–1000 nM). As shown, hsp90 dose-dependently enhanced nNOS activity with an EC_{50} of 24.1 $\pm$ 6.4 nM (mean $\pm$ S.E., $n = 3$). (**B**) Specific enhancing effect of hsp90 on nNOS activity. hsp90 (100 nM) significantly increased nNOS activity. In contrast, neither heat-denatured hsp90 (100 nM) nor the irrelevant protein BSA (100 nM) affected nNOS activity (**$P < 0.001$, compared with control, $n = 3$).

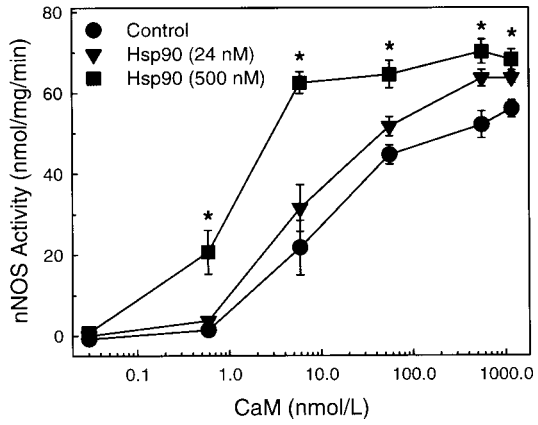

Figure 3 Effect of hsp90 on the CaM–nNOS dose–response relationship

Control CaM–nNOS dose–response curve was obtained with the presence of 1 mM Ca^{2+} and different concentrations of CaM (0–1000 nM). The CaM–nNOS dose–response curve was also determined with hsp90 (24 and 500 nM). In the presence of hsp90 (500 nM), the curve was shifted markedly to the left and the maximal activity was also increased (*$P < 0.05$, compared with control, $n = 6$).

treatment (68.2 $\pm$ 2.3 versus 56.0 $\pm$ 2.1 nmol/mg per min in the absence of hsp90, $P < 0.05$), suggesting that other mechanisms in addition to CaM binding might also participate in the action of hsp90.

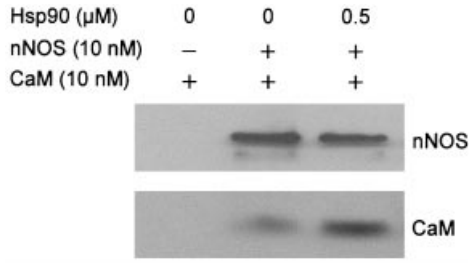

Figure 4 Enhancement of CaM binding to nNOS by hsp90

CaM and nNOS were incubated in buffer containing 50 mM Tris/HCl (pH 7.4), 125 mM NaCl, 1 mM Ca^{2+}, 100 μM L-arginine and 1 μM BH_4. After incubation, 2′,5′-ADP–Sepharose 4B resins were added. As shown, CaM itself did not bind with 2′,5′-ADP–sepharose resins (lane 1). Only the CaM bound with nNOS was detected by the resins (lane 2). With the same amount of nNOS, bound CaM was increased markedly in the presence of hsp90 (lane 3). These data are representative of three independent experiments.

To gain direct evidence that hsp90 may enhance CaM binding, an *in vitro* protein-binding assay was performed in the absence and presence of hsp90. As shown in Figure 4, CaM did not bind directly with 2′,5′-ADP–Sepharose 4B resins (Figure 4, lane 1). nNOS binds with 2′,5′-ADP–Sepharose 4B resins via its NADPH-binding sites. Therefore, the CaM detected from the resins was that bound with nNOS (Figure 4, lane 2). In the presence of hsp90 (0.5 μM), bound CaM was increased markedly, whereas the input of nNOS in each group remained the same (Figure 4, lane 3). These data demonstrate directly that hsp90 facilitated CaM binding to nNOS.

The above results demonstrate that hsp90 enhanced nNOS activity *in vitro*. This enzymic enhancement appeared to be a result of a direct allosteric interaction between nNOS and hsp90 because our measurements were conducted with purified protein preparations. hsp90 is one of the most abundant proteins in cells [16]. It is estimated that hsp90 constitutes 1–2% of total intracellular proteins and may reach micromolar cytosolic concentrations. Our data showed that hsp90 was a rather sensitive enhancer of nNOS with an approximate EC_{50} of 24 nM. This suggests strongly that the enhancement of nNOS activity by hsp90 occurs inside cells. Indeed, it was reported that hsp90 inhibition caused a decrease of NO production in nNOS-transfected cells [10].

Our findings from both enzymic measurement and protein-binding assay indicated that the potentiating effect of hsp90 on nNOS activity involved enhanced CaM binding. Although the molecular details of how hsp90 facilitates CaM binding to nNOS remain to be determined, a possible mechanism may be speculated upon in view of the biological roles of hsp90 in other well-established systems. Known as a molecular chaperone, hsp90 is involved in protein-refolding process and conformational regulation of the key proteins in signalling pathways such as steroid receptors and kinases [17]. Therefore, it is conceivable that the interaction with hsp90 may induce a change of nNOS conformation to a state more favourable for CaM binding.

Finally, we noticed that the maximal nNOS activity was also increased after hsp90 treatment. This suggested that beyond CaM-binding facilitation, additional mechanisms may be involved, and efforts to search for them are currently underway. Nevertheless, the findings described here demonstrate that hsp90 increases nNOS activity, at least partially, by enhancing CaM binding.

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