Inhibition of Superoxide Generation from Neuronal Nitric Oxide Synthase by Heat Shock Protein 90: Implications in NOS Regulation†

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ABSTRACT: Besides NO, neuronal NO synthase (nNOS) also produces superoxide ($O_2^{-\bullet}$) at low levels of L-arginine. Recently, heat shock protein 90 (hsp90) was shown to facilitate NO synthesis from eNOS and nNOS. However, the effect of hsp90 on the $O_2^{-\bullet}$ generation from NOS has not been determined yet. The interrelationship between its effects on $O_2^{-\bullet}$ and NO generation from NOS is also unclear. Therefore, we performed electron paramagnetic resonance measurements of $O_2^{-\bullet}$ generation from nNOS to study the effect of hsp90. Purified rat nNOS generated strong $O_2^{-\bullet}$ signals in the absence of L-arginine. In contrast to its effect on NO synthesis, hsp90 dose-dependently inhibited $O_2^{-\bullet}$ generation from nNOS with an IC_{50} of 658 nM. This inhibition was not due to $O_2^{-\bullet}$ scavenging because hsp90 did not affect the $O_2^{-\bullet}$ generated by xanthine oxidase. At lower levels of L-arginine where marked $O_2^{-\bullet}$ generation occurred, hsp90 caused a more dramatic enhancement of NO synthesis from nNOS as compared to that under normal L-arginine. Significant $O_2^{-\bullet}$ production was detected from nNOS even at intracellular levels of L-arginine. Adding hsp90 prevented this $O_2^{-\bullet}$ production, leading to enhanced nNOS activity. Thus, these results demonstrated that hsp90 directly inhibited $O_2^{-\bullet}$ generation from nNOS. Inhibition of $O_2^{-\bullet}$ generation may be an important mechanism by which hsp90 enhances NO synthesis from NOS.

Nitric oxide synthase (NOS)[1] plays a central role in the regulation of various biological processes including cardiovascular activity, neuronal signal transmission, and immune response (*1*, *2*). A family of NOS isoforms has been identified as neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). As implied by the definition, the prime function of NOS is to catalyze the reaction in which L-arginine and oxygen are converted to NO and L-citrulline (*3*−*5*). However, NO synthesis is certainly not the only function of NOS. It has become clear that all three NOSs also synthesize superoxide ($O_2^{-\bullet}$) under certain circumstances. At lower concentrations of L-arginine, nNOS produces $O_2^{-\bullet}$ in a Ca^{2+}/calmodulin (CaM)-dependent manner (*6*, *7*). In L-arginine-depleted cells, nNOS-derived $O_2^{-\bullet}$ reacts with NO to form another potent oxidant peroxynitrite leading to cellular injury (*8*). iNOS also produces $O_2^{-\bullet}$ under L-arginine depletion (*9*, *10*). Different from nNOS and iNOS, eNOS-mediated $O_2^{-\bullet}$ generation appeared to be primarily triggered and controlled by the cofactor tetrahydrobiopterin (BH_4) (*11*, *12*).

While the availability of enzyme substrates or cofactors can surely influence NO production, NO synthesis from NOS is mainly regulated by means of protein−protein interaction in cells (*5*, *13*). CaM is the chief modulator. Upon the rise of intracellular free [Ca^{2+}], CaM binds with nNOS and eNOS to initiate enzymatic activity. With a tightly bound CaM, iNOS is fully active under basal Ca^{2+} concentrations in resting cells (*14*). Beyond CaM, recent studies have revealed several other scaffolding proteins which critically modulate nNOS and eNOS activity. For example, nNOS was shown to target to membrane by interacting with postsynaptic density protein PSD-95 and PSD-93 in neurons (*15*). nNOS was also reported to bind with a 10 kDa protein PIN, and this binding inhibits nNOS activity (*16*). In endothelial cells, eNOS is localized in caveolae through coupling with caveolin-1 (*17*). This coupling results in inhibition of eNOS activity and a decrease in NO production. Recently, heat shock protein 90 (hsp90), a molecular chaperone, was shown to associate with eNOS and acted as an allosteric enhancer (*18*, *19*). Subsequent study suggested that hsp90 also facilitated nNOS activation in nNOS-transfected cells (*20*). However, the mechanism underlying the effect of hsp90 remains not completely understood. With purified nNOS, we demonstrated that hsp90 directly increased nNOS activity, and this appeared to be partially mediated by the enhancement of CaM binding affinity (*21*, *22*).

Most of the prior studies focused on the roles of the scaffolding proteins in regulating NO synthesis from NOSs. The effects of these proteins on the $O_2^{-\bullet}$ generation from NOSs were rarely investigated. Previous study using the hsp90 inhibitor geldanamycin suggested that inhibiting hsp90

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[1] Abbreviations: NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; hsp90, heat shock protein 90; CaM, calmodulin; EPR, electron paramagnetic resonance; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; L-NAME, *N*-nitro-L-arginine methyl ester; SOD, superoxide dismutase.

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function may induce $O_2^{-\bullet}$ formation from eNOS in cultured endothelial cells (*23*). However, recent study raised concern on the use of geldanamycin to study the effect of hsp90 on $O_2^{-\bullet}$ generation from NOS because geldanamycin may directly redox-cycle with flavin sites of NOS leading to hsp90-independent $O_2^{-\bullet}$ formation (*24*). Therefore, it remains unclear whether and how hsp90 modulates $O_2^{-\bullet}$ generation from NOS. It is also not known if the effect of hsp90 on $O_2^{-\bullet}$ generation from NOS mediates its enhancement of NO synthesis. In the present study, we directly measured $O_2^{-\bullet}$ generation from purified nNOS using electron paramagnetic resonance (EPR) spectroscopy to explore the effect of hsp90. We found that hsp90 directly inhibited $O_2^{-\bullet}$ generation from nNOS. Our results also suggested that inhibition of $O_2^{-\bullet}$ generation may contribute to the enhancing action of hsp90 on NO synthesis from nNOS.

MATERIALS AND METHODS

Materials. Cell culture materials were obtained from Gibco BRL (Gaithersburg, MD). Bovine hsp90, greater than 95% pure, was purchased from Sigma Chemical Co. (St. Louis, MO). 2′,5′-ADP−Sepharose 4B was the product of Pharmacia Biotech. Inc. (Piscataway, NJ). L-[^{14}C]Arginine was purchased from DuPont/NEN (Boston, MA). 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO, >99% pure) was the product of Oxis International Inc. (Portland, OR). The xanthine oxidase, superoxide dismutase (SOD), CaM, NADPH, L-arginine, BH_4, *N*-nitro-L-arginine methyl ester (L-NAME), and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

nNOS Purification. Recombinant rat nNOS was isolated from stably transfected human embryonic kidney 293 cells (from Dr. Solomon H. Snyder at Johns Hopkins University School of Medicine). The purification protocol was slightly modified from the procedure described before (*21, 25*). In brief, nNOS-transfected 293 cells were grown in minimum essential medium (MEM) with 10% heat-inactivated fetal calf serum. Cells were harvested in phosphate-buffered saline and pelleted by a brief spinning. The cell pellets were then homogenized and sonicated in buffer A (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol). After centrifugation (16000*g*, 10 min) at 4 °C, supernatants of the homogenates were loaded onto a 2′,5′-ADP−Sepharose 4B column preequilibrated in buffer A. The column was washed with 50 mL of buffer A containing 450 mM NaCl twice and followed by 10 mL of Tris-HCl buffer (50 mM, pH 7.4) 3 times. Then the bound 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 bovine serum albumin as standard. The purity of nNOS was determined by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and visualized with Coomassie 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.

In Vitro Protein Binding Assay. The hsp90 bound with nNOS was detected using the NOS-specific affinity resin 2′,5′-ADP−Sepharose (*21*). In brief, the binding between nNOS and hsp90 was conducted in 500 μL of binding buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.5 mM Ca^{2+}, 0.5% NP-40, 15 nM nNOS, and hsp90 (0.15−1.5 μM). After 2 h incubation at 4 °C, 2′,5′-ADP−Sepharose resins (50 μL of 50% slurry) were added, and the mixtures were incubated overnight at 4 °C. After binding, the resins were extensively washed (6 times) by high-salt buffer (50 mM Tris-HCl, pH 7.4, 500 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. Hsp90 and nNOS were identified by Western blotting using anti-hsp90 and anti-nNOS monoclonal antibodies (Transduction Laboratory).

EPR Spectroscopy and Spin Trapping. Spin-trapping measurements of oxygen free radicals were performed in 500 μL of buffer containing 50 mM Tris-HCl (pH 7.4), 0.5 mM NADPH, 0.5 mM Ca^{2+}, 10 μg/mL CaM, 15 nM purified nNOS, and 20 mM spin trap DEPMPO. In the experiments determining the effect of hsp90, nNOS and hsp90 were incubated for 15 min prior to measurements. EPR spectra were recorded in a quartz flat cell at room temperature (23 °C) with a Bruker ER 300 spectrometer operating at X-band with a TM 110 cavity using a modulation frequency of 100 kHz, modulation amplitude of 0.5 G, microwave power of 20 mW, and microwave frequency of 9.785 GHz. The microwave frequency and magnetic field were precisely measured using an EIP 575 microwave frequency counter and Bruker ER 035 NMR gaussmeter. The observed $O_2^{-\bullet}$ signals were quantitated by double integration as previously described (*8*−*10, 12*).

L-[^{14}C]Arginine to L-[^{14}C]Citrulline Conversion Assay. nNOS-catalyzed L-[^{14}C]arginine to L-[^{14}C]citrulline conversion was monitored 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+}, 10 μg/mL CaM, 1 μM BH_4, and 8 nM nNOS. In some measurements, additional L-arginine (100 μM) was included. To determine the effects of hsp90, nNOS was preincubated with different concentrations of hsp90 for 15 min. The reaction was initiated by adding nNOS and terminated after 2 min incubation (linear range of L-citrulline formation) 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 quantitated by liquid scintillation counting (*26*).

Statistics. Data were expressed as mean ± SE. 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

Recombinant rat nNOS was isolated from the stably transfected HEK 293 cells by affinity chromatography. The purity and catalytic activity of the nNOS preparations were analyzed. As shown in Figure 1A, purified nNOS preparations displayed one prominent band (>90%) on SDS−PAGE with a molecular mass of 160 kDa, which was in agreement with the molecular mass of native nNOS reported previously (*3*−*5*). Purified nNOS preparations exhibited strong catalytic activity (656 ± 58.6 nmol mg^{-1} min^{-1}) as measured by the L-[^{14}C]arginine to L-[^{14}C]citrulline conversion assay (Figure

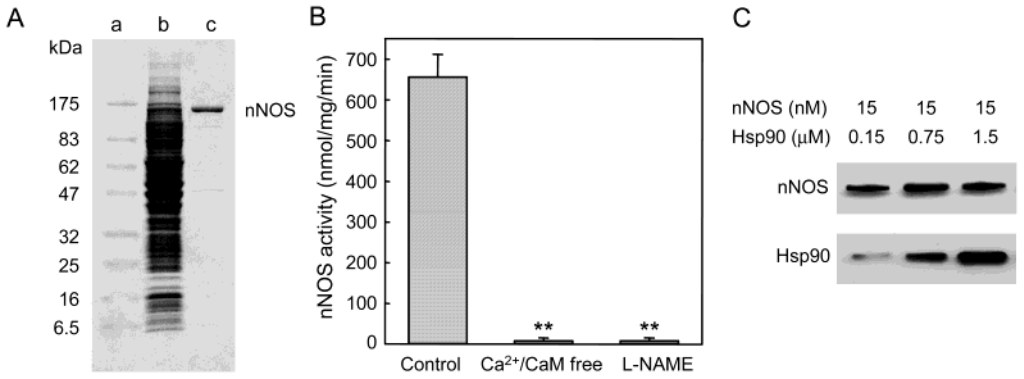

FIGURE 1: Characterization of recombinant rat nNOS purified from stably transfected HEK 293 cells. (A) SDS−polyacrylamide gel electrophoresis analysis of isolated nNOS preparations. Lane a, molecular mass markers; b, cell homogenates; c, purified nNOS eluted from affinity columns. Proteins were separated on 4−20% gradient gels and visualized by Coomassie blue staining. (B) Catalytic activity of purified nNOS preparations. NOS activity was measured by the L-[^{14}C]arginine to L-[^{14}C]citrulline conversion assay. The preparations exhibited typical constitutive nNOS characteristics with L-NAME (1 mM) inhibitory and Ca^{2+}/CaM-dependent activity (mean ± SE; **, $P < 0.01$, vs control; $n = 6$). (C) Association of hsp9 with nNOS. The hsp90 bound with nNOS was pulled down using nNOS affinity resin 2′,5′-ADP−Sepharose. In the presence of an equal amount of nNOS, adding more hsp90 (0.15−1.5 μM) significantly increased the amounts of hsp90 associated with nNOS.

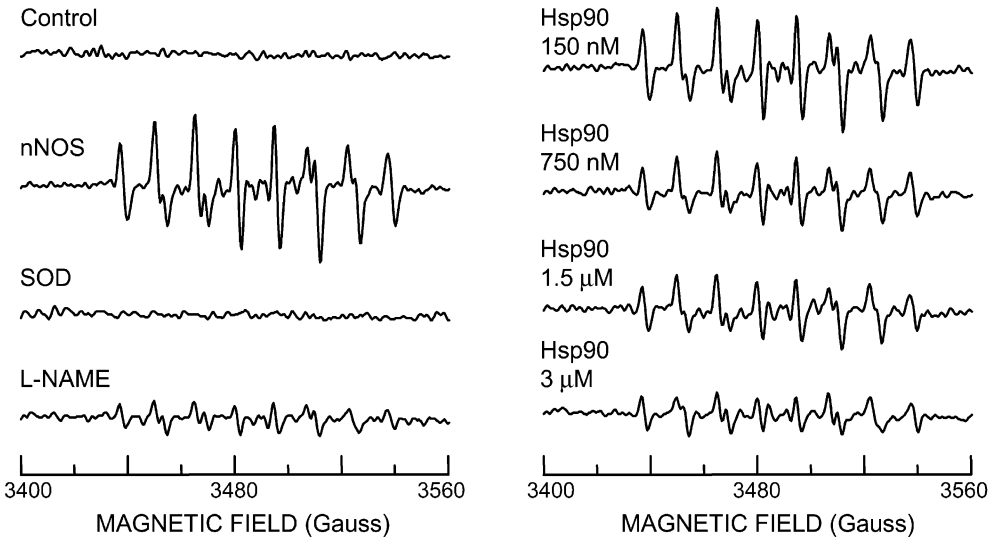

FIGURE 2: EPR spectra of $O_2^{-\bullet}$ generated by nNOS in the absence and presence of hsp90. EPR measurements were performed in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM NADPH, 0.5 mM Ca^{2+}, 10 μg/mL CaM, and 20 mM DEPMPO. While no signal was detected in the reaction system without enzyme (Control), strong signals were seen after adding nNOS (15 nM). The prominent signals displayed the characteristic DEPMPO-OOH spectrum. A small DEPMPO-OH signal was also observed. These signals were quenched by SOD (200 units/mL). The NOS inhibitor L-NAME (1 mM) also blocked this $O_2^{-\bullet}$ production. Hsp90 dose-dependently decreased these $O_2^{-\bullet}$ signals. EPR spectra were recorded at room temperature with a microwave frequency of 9.785 GHz, microwave power of 20 mW, and 0.5 G modulation amplitude. Representative spectra shown were from three independent measurements.

1B). This catalytic activity was abolished by the specific NOS inhibitor L-NAME (1 mM), confirming which was derived from nNOS. NOS activity also required the addition of Ca^{2+}/CaM, a characteristic of constitutive nNOS. Protein binding experiments further proved that hsp90 associated with nNOS (Figure 1C). With the presence of an equal amount of nNOS, adding more hsp90 (0.15−1.5 μM) markedly increased the amounts of hsp90 bound with nNOS.

To determine the effect of hsp90 on $O_2^{-\bullet}$ generation from nNOS, EPR spin-trapping measurements were conducted on nNOS in the absence of L-arginine using the recently developed spin-trap DEPMPO. DEPMPO traps $O_2^{-\bullet}$ to form a stable spin adduct, DEPMPO-OOH, which enables accurate measurements of $O_2^{-\bullet}$ formation (*27, 28*). In the control experiments without nNOS, no signal was detected from the system containing NADPH and Ca^{2+}/CaM (Figure 2, Control). After adding nNOS (15 nM), strong EPR signals were seen (Figure 2, nNOS). These prominent signals exhibited the characteristic DEPMPO-OOH spectrum, indicative of trapped $O_2^{-\bullet}$. A small DEPMPO-OH signal was also observed. These signals were abolished by SOD (200 units/mL) (Figure 2, SOD), proving that $O_2^{-\bullet}$ was the primary oxygen free radical generated. These signals were also blocked by L-NAME (1 mM) (Figure 2, L-NAME), confirming that nNOS was responsible for $O_2^{-\bullet}$ formation. To study the effect of hsp90, nNOSs were preincubated with hsp90, and then $O_2^{-\bullet}$ production was measured. As shown, hsp90 inhibited $O_2^{-\bullet}$ generation from nNOS in a dose-dependent manner (Figure 2, Hsp 150 nM to 3 μM). The IC_{50} for hsp90 on $O_2^{-\bullet}$ generation from nNOS was approximately 658 nM (Figure 3, left panel). We also determined the effect of hsp90 on the time course of $O_2^{-\bullet}$ generation by nNOS. In the control experiments without hsp90, a typical time course of $O_2^{-\bullet}$ formation was obtained

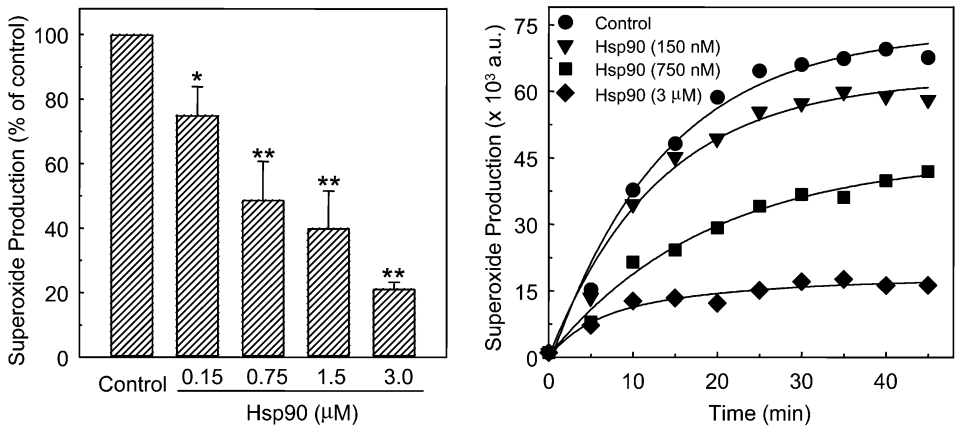

FIGURE 3: Effect of hsp90 on $O_2^{-\bullet}$ generation from nNOS. Left panel: Hsp90 inhibited $O_2^{-\bullet}$ generation from nNOS in a dose-dependent manner. Data are shown as mean ± SE (*, $P < 0.05$; **, $P < 0.01$ vs control; $n = 3$). Right panel: Effect of hsp90 on the time course of $O_2^{-\bullet}$ generation from nNOS. Spectra were continuously recorded at five 1-min acquisitions from the beginning of the reaction until 45 min. These results are representatives of triplicate experiments.

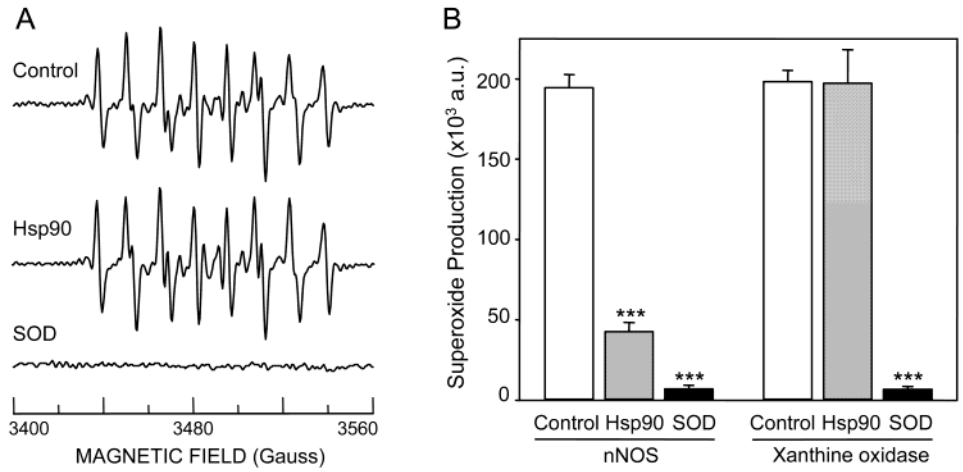

FIGURE 4: Effect of hsp90 on the $O_2^{-\bullet}$ generated by xanthine oxidase. (A) EPR spectra of $O_2^{-\bullet}$ generation from xanthine oxidase. The reaction system consisted of 0.05 unit/mL xanthine oxidase, 0.5 mM xanthine, and 20 mM DEPMPO in 50 mM Tris-HCl buffer (pH 7.4). Strong $O_2^{-\bullet}$ signals were measured from xanthine oxidase (Control). Hsp90 (3 μM) had no effect on the $O_2^{-\bullet}$ signals generated by xanthine oxidase. In contrast, SOD (200 units/mL) completely abolished the $O_2^{-\bullet}$ spectrum. These spectra are representatives of three independent experiments. (B) Comparison of the effects of hsp90 and SOD on the $O_2^{-\bullet}$ generation from xanthine oxidase and nNOS (mean ± SE; ***, $P < 0.001$ compared with control groups; $n = 3$).

from nNOS (Figure 3, right panel). Hsp90 dose-dependently decreased the magnitude of $O_2^{-\bullet}$ generation from nNOS as a function of time. With the presence of 3 μM hsp90, the time course of $O_2^{-\bullet}$ production by nNOS was largely blocked.

To investigate whether the inhibitory effect of hsp90 on $O_2^{-\bullet}$ formation was due to its direct action on nNOS or through $O_2^{-\bullet}$ scavenging, the effect of hsp90 on the $O_2^{-\bullet}$ generation from xanthine oxidase was examined. In the absence of hsp90, xanthine oxidase produced strong $O_2^{-\bullet}$ signals (Figure 4A, Control). Then xanthine oxidase was preincubated with 3 μM hsp90, a concentration known to largely inhibit $O_2^{-\bullet}$ formation by nNOS (Figure 3). To achieve a quantitative comparison, $O_2^{-\bullet}$ generation from xanthine oxidase was adjusted to the level similar to that from nNOS (Figure 4B). In contrast to its effect on nNOS, hsp90 did not affect the $O_2^{-\bullet}$ generated by xanthine oxidase (Figure 4A, Hsp90). On the other hand, the genuine $O_2^{-\bullet}$ scavenging enzyme SOD totally quenched the $O_2^{-\bullet}$ signals generated by either xanthine oxidase or nNOS (Figure 4A, SOD; Figure 4B). These results demonstrated that hsp90 inhibited $O_2^{-\bullet}$ generation from nNOS by a direct interaction with the enzyme but not through $O_2^{-\bullet}$ scavenging.

To explore whether inhibition of $O_2^{-\bullet}$ generation by hsp90 contributed to its potentiating effect on NO synthesis from nNOS, we compared the effects of hsp90 on nNOS-catalyzed L-citrulline formation under different levels of L-arginine. Our recent studies showed that hsp90 may enhance CaM biding affinity to nNOS (*21, 22*). The following experiments were therefore carried out with a saturated amount of CaM (10 μg/mL) to rule out the effect caused by altered CaM binding. In the presence of higher levels of L-arginine (100 μM) where less $O_2^{-\bullet}$ was produced, hsp90 dose-dependently induced a moderate but significant increase of nNOS activity (162 ± 10.2% of the control activity at 0.8 μM hsp90, $P < 0.001$, compared to the control without hsp90) (Figure 5, open circles). We hypothesized that this portion of NOS enhancement by hsp90 may be attributed to its prevention of $O_2^{-\bullet}$ release from nNOS. Since nNOS produces more $O_2^{-\bullet}$ at lower levels of L-arginine, this hypothesis will predict a more dramatic enhancement of nNOS activity by hsp90 at lower L-arginine concentrations. Indeed, under lower levels

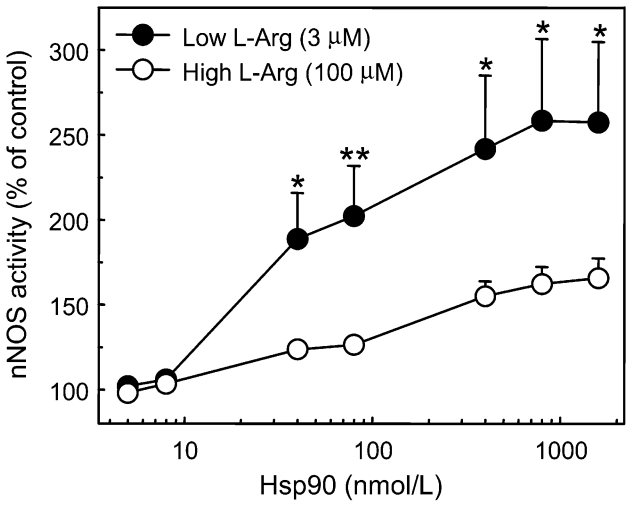

FIGURE 5: Enhancement of nNOS activity by hsp90 under different levels of L-arginine. NOS activity was assayed by monitoring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. The reaction was conducted in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM NADPH, 1 mM Ca^{2+}, 10 μg/mL CaM, 1 μM BH_4, 3 μM L-[^{14}C]-arginine, and 8 nM nNOS. In the experiments determining the effect of hsp90 under higher L-arginine level (100 μM), additional L-arginine was included (mean ± SE; *, $P < 0.05$; **, $P < 0.01$ compared with high L-arginine group; $n = 6-9$).

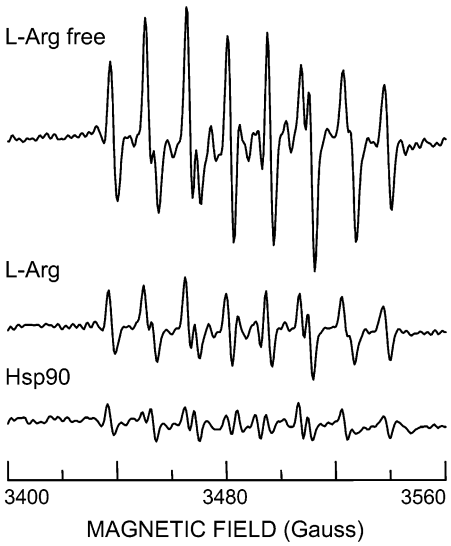

FIGURE 6: nNOS-catalyzed $O_2^{-\bullet}$ generation in the presence of intracellular levels of L-arginine. EPR measurements of $O_2^{-\bullet}$ generation from nNOS were conducted in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM NADPH, 0.5 mM Ca^{2+}, 1 μM BH_4, 10 μg/mL CaM, and 20 mM DEPMPO. In the absence of L-arginine, nNOS (15 nM) produced strong $O_2^{-\bullet}$ signals. In the presence of 100 μM L-arginine, a concentration compatible to the cytosolic free L-arginine content, significant $O_2^{-\bullet}$ signals were still seen. Adding hsp90 (1.5 μM) largely prevented this $O_2^{-\bullet}$ production. Representative spectra are shown from two independent experiments.

of L-arginine (3 μM), hsp90 enhanced nNOS activity to a greater extent (Figure 5, filled circles). Hsp90 (0.8 μM) induced a 258 ± 48.3% increase in nNOS activity under lower L-arginine concentrations as compared to a 162 ± 10.2% increase at higher L-arginine levels ($P < 0.05$). These data were consistent with the hypothesis that prevention of $O_2^{-\bullet}$ generation contributes to the enhancing effect of hsp90 on nNOS activity.

Finally, we sought to prove that $O_2^{-\bullet}$ generation from nNOS occurred in the presence of intracellular levels of L-arginine and examine if hsp90 can prevent it. As shown in Figure 6, while decreased as compared with that under L-arginine-free conditions, significant $O_2^{-\bullet}$ signals were still detected from nNOS even at high L-arginine concentration (100 μM), a concentration compatible to intracellular free L-arginine content (*8, 29*). Adding hsp90 largely blocked this $O_2^{-\bullet}$ production. These results demonstrated that certain amounts of $O_2^{-\bullet}$ generation occurred from nNOS in the presence of normal intracellular L-arginine concentrations; however, hsp90 can markedly inhibit it.

DISCUSSION

The fundamental finding of the current study was that hsp90 inhibited $O_2^{-\bullet}$ generation from nNOS. $O_2^{-\bullet}$ generation is a general feature of all NOS isoforms (*6, 10−12*). Previous studies showed that the availability of the enzyme substrate L-arginine or cofactor BH_4 plays important roles in controlling the onset of $O_2^{-\bullet}$ synthesis from NOSs (*6−12*). The present study identified hsp90 as a novel endogenous protein inhibitor for nNOS-mediated $O_2^{-\bullet}$ generation. Hsp90 is known as one of the most abundant cytosolic proteins in eukaryotic cells under normal conditions (*30*). In most cells, hsp90 was estimated to account for 1−2% of the total cellular protein and may reach micromolar intracellular concentrations. Under stress conditions, its expression level can further increase severalfold (*31*). Our in vitro measurements showed that hsp90 dose-dependently inhibited $O_2^{-\bullet}$ production by nNOS with an approximate IC_{50} of 658 nM. The fact that hsp90 is highly abundant in cytosol suggested that inhibition of $O_2^{-\bullet}$ generation from nNOS by hsp90 may also occur inside cells.

Hsp90 inhibited $O_2^{-\bullet}$ production from nNOS but not that from the well-characterized $O_2^{-\bullet}$-generating enzyme xanthine oxidase. On the other hand, SOD quenched the $O_2^{-\bullet}$ generated by either nNOS or xanthine oxidase. These data indicated that hsp90 blocked $O_2^{-\bullet}$ generation from nNOS not through $O_2^{-\bullet}$ scavenging. Since this study was conducted with purified nNOS and hsp90, inhibition of $O_2^{-\bullet}$ formation was likely due to the direct interaction between nNOS and hsp90. Though the detailed molecular mechanism regarding how exactly hsp90 prevents nNOS from generating $O_2^{-\bullet}$ remains to be uncovered, certain possibilities may be speculated in light of the roles of hsp90 in other well-characterized systems. Known as a molecular chaperone, hsp90 participates in protein refolding and conformational regulation of steroid receptors and kinases (*32−34*). Conceivably, the interaction with hsp90 may keep nNOS in a conformation prohibiting $O_2^{-\bullet}$ formation and favoring NO synthesis.

While hsp90 was shown to enhance NO synthesis from eNOS and nNOS, the mechanism for its action has been elusive. Our recent enzymatic assay and protein binding experiments showed that increased CaM binding affinity to nNOS was involved in the effect of hsp90 (*21, 22*). Interestingly, hsp90 not only shifted the CaM−nNOS dose−response curve to the left but also enhanced the maximal activity. This suggested that in addition to CaM affinity enhancement, other mechanisms also exist in the action of hsp90. Indeed, even in the presence of a saturated amount of CaM, hsp90 can still significantly increase nNOS activity (Figure 5). The

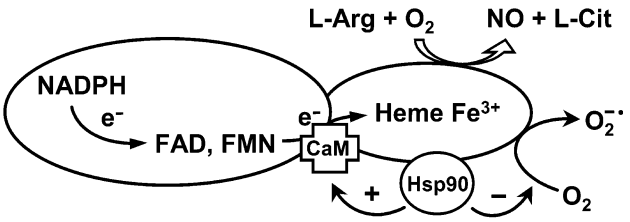

FIGURE 7: Schematic diagram illustrating the mechanisms for the enhancement of hsp90 on NO synthesis from nNOS. nNOS is a bidomain enzyme comprising a C-terminal reductase and N-terminal oxygenase domain. NO synthesis involves an electron transfer from NADPH bound at the reductase domain to the heme center of the oxygenase domain (denoted by the arrows). CaM binding facilitates electron transfer between the two domains. Augmentation of CaM binding affinity is one mechanism for the enhancing action of hsp90 on NO synthesis (*21*, *22*). In addition to NO, nNOS also produces certain amounts of $O_2^{-\bullet}$. $O_2^{-\bullet}$ and its associated reactive oxygen species can damage nNOS function. Hsp90 inhibits this $O_2^{-\bullet}$ generation and prevents nNOS from oxidant damage. Therefore, inhibition of $O_2^{-\bullet}$ generation is another possible mechanism for the enhancement of hsp90 on NO synthesis from nNOS.

present study suggested that inhibition of $O_2^{-\bullet}$ release from nNOS may be a possible mechanism. It has been well established that $O_2^{-\bullet}$ as well as its associated reactive oxygen species damage enzyme function (*35*, *36*). In particular, $O_2^{-\bullet}$ can react with NO to form a more potent oxidant peroxynitrite. Peroxynitrite has been known to be extremely harmful to enzymes (*37*−*39*). $O_2^{-\bullet}$ and peroxynitrite can damage NOS function by either directly destroying enzyme structure or oxidizing the critical enzyme cofactor such as BH_4. Inhibition of $O_2^{-\bullet}$ generation by hsp90 should therefore be beneficial to nNOS function.

We have designed two experimental conditions to test the above hypothesis. In the presence of higher levels of L-arginine, limited $O_2^{-\bullet}$ formation can only cause small damage to the enzyme. In correspondence, inhibition of this small $O_2^{-\bullet}$ production by hsp90 is anticipated to result in a moderate increase nNOS activity. Under lower levels of L-arginine, large amounts of $O_2^{-\bullet}$ will be generated and can severely damage nNOS function. Adding hsp90 blocks this large $O_2^{-\bullet}$ production and should accordingly increase nNOS activity to a higher extent. Our results from both enzymatic assay and EPR $O_2^{-\bullet}$ measurements were consistent with these predictions. Hsp90 indeed induced a significantly greater enhancement of nNOS activity at lower levels of L-arginine. EPR measurements also reconfirmed that nNOS produced more $O_2^{-\bullet}$ at lower L-arginine concentrations. Taken together, we propose that in addition to facilitating CaM binding, $O_2^{-\bullet}$ inhibition may be another mechanism for the enhancing action of hsp90 on NO synthesis from nNOS (Figure 7).

The biological function of NOS is primarily conveyed by its product NO (*1*−*5*). It is a paradox that NOSs are also capable of producing large amounts of $O_2^{-\bullet}$ because the latter readily inactivates NO to form the more harmful peroxynitrite. How does the cell cope with this apparently self-destructive dilemma? Our current study along with previous studies from others suggested that at least two mechanisms may be involved. First, the intracellular L-arginine and BH_4 content may limit $O_2^{-\bullet}$ generation from nNOS at low levels (*7*, *8*). However, as demonstrated by the present study, cytosolic L-arginine concentrations were not sufficient to completely eliminate $O_2^{-\bullet}$ generation from nNOS. Significant amounts of $O_2^{-\bullet}$ were still detected from nNOS even under intracellular levels of L-arginine (Figure 6). Therefore, additional mechanisms are needed to further restrain $O_2^{-\bullet}$ formation. Our current findings suggested a novel mechanism by means of protein−protein interaction. Hsp90 interacted with nNOS, leading to $O_2^{-\bullet}$ inhibition. Accordingly, NO synthesis from nNOS was enhanced. To our knowledge, this is the first example that NOS activity was found to be modulated by an intracellular chaperone protein through intervening in its $O_2^{-\bullet}$ generation process.

In summary, our study has identified hsp90 as an important endogenous protein inhibitor of $O_2^{-\bullet}$ generation from nNOS. Coupling between hsp90 and nNOS is therefore important for normal nNOS function. Disruption of this coupling may result in $O_2^{-\bullet}$ overproduction, which could in turn lead to nNOS dysfunction and disease.

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