Heat Shock Protein 90 Mediates the Balance of Nitric Oxide and Superoxide Anion from Endothelial Nitric-oxide Synthase*

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The balance of nitric oxide (NO) and superoxide anion (O2−) plays an important role in vascular biology. The association of heat shock protein 90 (Hsp90) with endothelial nitric-oxide synthase (eNOS) is a critical step in the mechanisms by which eNOS generates NO. As eNOS is capable of generating both NO and O2−, we hypothesized that Hsp90 might also mediate eNOS-dependent O2− production. To test this hypothesis, bovine coronary endothelial cells (BCEC) were pretreated with geldanamycin (GA), 10 μg/ml; 17.8 μM) and then stimulated with the calcium ionophore, A23187 (5 μM). GA significantly decreased A23187-stimulated eNOS-dependent nitrite production (p < 0.001, n = 4) and significantly increased A23187-stimulated eNOS-dependent O2− production (p < 0.001, n = 8). A23187 increased phospho-eNOS(Ser-1179) levels by >1.6-fold over vehicle (V)-treated levels. Pretreatment with GA by itself or with A23187 increased phospho-eNOS levels. In unstimulated V-treated BCEC cultures low amounts of Hsp90 were found to associate with eNOS. Pretreatment with GA and/or A23187 increased the association of Hsp90 with eNOS. These data show that Hsp90 is essential for eNOS-dependent NO production and that inhibition of ATP-dependent conformational changes in Hsp90 uncoouples eNOS activity and increases eNOS-dependent O2− production.

Nitric oxide (NO) and superoxide anion (O2−) play opposing roles in vascular biology. Nitric oxide generation is increased greatly when Hsp90 associates with eNOS in endothelial cell cultures (1, 2). A decrease in the amount of Hsp90 co-purifying with eNOS is associated with a decrease in NO production by pulmonary artery endothelial cells exposed to prolonged periods of hypoxia (3). Geldanamycin (GA) is an ansamycin antibiotic that binds to the ATP binding site of Hsp90, thereby inhibiting the ATP/ADP cycle required for the interaction with client proteins such as eNOS (2–4). GA has been used to demonstrate that NO production in mesenteric arteries and rat aortas depends on Hsp90 activity, implying that factors adversely affecting this interaction between Hsp90 and eNOS may be one of the mechanisms for portal hypertension and increased vascular tone (2, 4). Taken together, these reports indicate that Hsp90 is critical for eNOS generation of NO.

 Emerging evidence suggests that under pathological conditions eNOS may also generate O2− (5–7). Electron spin resonance studies clearly demonstrate that purified recombinant eNOS generates O2− when activated by calmodulin (CaM) in the absence of its substrate, l-arginine, or the essential cofactors, tetrahydrobiopterin (8, 9). As Hsp90 is important for mediating eNOS-dependent NO generation we wondered whether Hsp90 also mediates eNOS O2− generation. Our findings indicate that inhibiting Hsp90 function uncouples eNOS activity to increase eNOS-dependent O2− generation.

MATERIALS AND METHODS

1-Nitroargininemethylester (l-NAME), NG-monomethyl-l-arginine-monooacetate, (l-NMA), dimethyl sulfoxide (MeSO), geldanamycin (GA), antibiotics/mycotics, trypsin-EDTA, Hank’s balanced salts solution (HBSS), l-arginine, KI, glacial acetic acid, sodium nitrite, ferricytochrome c, superoxide dismutase (SOD), NaF, sodium deoxycholate, SDS, 4-(2-aminoethyl benzene) sulfonyl fluoride hydrochloride, sodium orthovanadate, leupeptin, pepstatin A, aprotinin, protein A-Sepharose were from Sigma Chemical Company (St. Louis, MO). l-Sepiapterin was from Alexis Biochemicals (San Diego, CA). (HCl, NaOH, were from Fisher Scientific (Pittsburg, PA). RPMI 1640 was from Life Technologies, Inc. (Grand Island, NY). Tris-HCl and NaCl were from Baker (Phillipsburg, NJ). Hydrocholide was from Poly- sciences, Inc. (Warrington, PA). Triton X-100 was from Lab Chem (Pittsburg, PA). A23187 was from CalBiochem (San Diego, CA). Lactomyl buffer, polyacrylamide, nitrocellulose membranes were from Bio-Rad (Hercules, CA). Fetal bovine serum was from HyClone (Logan, UT). ECL reagents were from Amersham Pharmacia Biotech. X-OMAT film was from Kodak (Rochester, NY). H32 antibody was from BioMol (Plymouth Meeting, PA) and Jennifer Pollock (Medical College of Georgia, Augusta, GA). Anti-phospho-eNOS (Ser-1177) was from Cell Signaling Technology (Beverly, MA).

Endothelial Cell Culture—Bovine coronary endothelial cells (BCEC) were provided by William B. Campbell (Milwaukee, WI). BCEC were cultured in RPMI 1640 media containing 20% fetal bovine serum, antibiotics, mycotics, rHGF (10 ng/ml), and heparin (5 units/ml). BCEC were passaged with trypsin-EDTA and used for experiments between passage 5–7.

Experimental Protocol—The protocol for the experiments was as follows. For nitrite and O2− studies, l-NMA and l-NAME (1 mM), respectively were added to confluent BCEC cultures at time 0. At 30 min, vehicle (V is dimethyl sulfoxide) or GA (10 μg/ml or 17.8 μM) was added

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1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; GA, geldanamycin; BCEC, bovine coronary endothelial cells; PAGE, polyacrylamide gel electrophoresis; HBP, horseradish peroxidase; l-NAME, l-nitroargininemethylester, l-NMA, NG-monomethyl-l-arginine-monooacetate; SOD, superoxide dismutase; V, vehicle; RIPA, radiommune precipitation buffer.
to the BCEC cultures and the l-NAME- or l-NMA-treated BCEC cultures. At 60 min, the four test groups were washed three times with HBSS and then analyzed in separate experiments for nitrite production and release of $O_2^-$ to examine the role of tetrahydrobiopterin, BCEC cultures were incubated with sepiapterin (100 μM) for 30 min prior to incubation with V, GA, and l-NAMe in the O2 protocol. For the co-precipitation studies, substrate analog inhibitors of eNOS were not included in the protocols.

**Measurements of Nitrite**—After the third wash, V-treated, l-NMA-treated, GA-treated, and l-NMA-treated test cultures in 6-well plates were incubated with 0.75 ml HBSS containing A23187 (5 μM) and l-arginine (25 mM) for 30 min. Nitrites were quantified using the Griess reaction. Further details of these studies are described (10). Each experiment was performed in triplicate; nitrites were analyzed in duplicate or triplicate, and cell protein for each well was determined in duplicate.

**Superoxide Anion**—After the final HBSS wash, the test groups in 6-well dishes (V, l-NAME, GA, and l-NAMe) were incubated with 1 ml of HBSS containing ferricytochrome c (50 μM) and A23187 (5 μM) with and without l-NAMe (1 mM) for 30 min. Superoxide anion production was calculated from the absorbance of ferricytochrome c at 550 nm. The release of $O_2^-$ was calculated from the molar extinction coefficient ($\varepsilon = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$). Data were compared with $O_2^-$ release from independent wells incubated with HBSS containing ferricytochrome c and SOD, 1000 units/ml. Each experiment was performed in triplicate; protein from each well was analyzed in duplicate.

**Detection of Endothelial Superoxide Anion Generation in Isolated Carotid Arteries**—Canine carotid arteries were obtained from adult mongrel dogs. The vessel was removed, transferred to a physiologic saline solution and adventitia was cleared. The artery was sectioned into segments at least 2 cm long and placed in RPMI 1640 media. After two washings in RPMI 1640 (to remove adherent blood cells), vessels were placed in organ culture. Artery segments were incubated with V or GA as above, washed free of vehicle and GA, and then incubated with hydroethidine (10 μM) for 30 min. Hydroethidine is taken up by cells and in the presence of $O_2^-$ is converted to ethidine, which intercalates with nuclear DNA. The degree of fluorescence is proportional to the amount of $O_2^-$ present (11). In situ fluorescence was assessed using confocal microscopy.

**Western Analysis, Immunoprecipitation, and Immunoblotting**—Phospho-eNOS(Ser-1177) and eNOS levels were determined using the manufacturer’s protocol. Briefly, confluent BCEC cultures in 60-mm dishes were pretreated with GA (10 μg/ml) for 30 min. The cultures were washed three times with HBSS and then stimulated with A23187 (5 μM) in HBSS for 10 min at 37 °C with gentle horizontal rotation (50 rpm). After preincubation with GA, the test groups were washed three times with HBSS and then incubated at 37 °C in tissue culture incubator in 5 ml of HBSS containing l-arginine (10 μM) with and without A23187 (5 μM). After 10 min, the HBSS solutions were removed by aspiration, and cell proteins were harvested in 200 μl of SDS sample buffer. Aliquots (50 μl) were heated (95 °C, 5 min) and stored on ice until loaded (20 μl/ lane) on 7% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and blotted with anti-phospho-eNOS(Ser-1177) and anti-eNOS (9D10, Zymed Laboratories Inc.) overnight at 4 °C. Bands were visualized using the appropriate horseradish peroxidase-linked secondary antibodies and ECL reagents.

**Co-precipitation studies** for determining the interaction of Hsp90 with eNOS were performed on confluent BCEC cultures in 100-mm dishes. The four test groups were V, GA, V+A23187, and GA+A23187. After preincubation with GA, the test groups were washed three times with 5 ml of HBSS and then incubated at 37 °C in tissue culture incubator for 15 min in 5 ml of HBSS containing l-arginine (10 μM) with and without A23187 (5 μM). After 10 min, the HBSS solutions were removed, and the cells were lyzed in modified RIPA buffer as described (2). The samples were sonicated two times at a power setting of 1.25–1.5 for 30 s on a Fisher Scientific Dismembrater (Model 550) fitted with an ultrasonic Transducer (Model No. CLA4 with a frequency of 20 kHz) probe. For this procedure it is important to adjust the power settings of the unit to 5–10% of maximum power output because higher power outputs disrupt eNOS interactions with other cell proteins. Cell debris was removed from the cell lysates, and eNOS was immunoprecipitated as described (2). The immunoprecipitated proteins were separated by SDS-PAGE (7.5–15%) and transblotted onto nitrocellulose. The membrane was blocked with 5% nonfat milk in TBS-Tween (0.1%) and then incubated for 1 h with either anti-Hsp90 from Sigma using ECL reagents and Kodak X-OMAT film. The autoradiograms were imaged with Adobe PhotoShop v3.5 and UMAX Magiscan v4.4 software, and the relative band densities were quantified using NIH Image 1.62.

**Statistical Analysis**—Nitrite and superoxide anion data were examined by one-way analysis of variance to determine whether the variances and means were significantly different. The Newman-Kuel post-hoc test was employed to determine the level of significance between means ($***, p < 0.001$ and $*, p < 0.01$).

**RESULTS**

Geldanamycin (GA) significantly inhibited A23187-stimulated nitrite production by BCEC cultures incubated in HBSS containing low concentrations of l-arginine (25 μM) (Fig. 1A). In contrast, A23187 stimulation of GA-treated BCEC cultures significantly increased the release of $O_2^-$ compared with vehicle (V)-treated BCEC cultures (Fig. 1B). l-NAME significantly reduced $O_2^-$ release from GA-treated cells (Fig. 1B) but did not inhibit the release of $O_2^-$ from V-treated cells. These data indicate that when NO production is inhibited by GA, eNOS generates $O_2^-$. These findings are similar to previous studies showing that l-NAME inhibited the release of $O_2^-$ from endothelial cells incubated with native or oxidized low-density lipoprotein (5, 6). When the production of $O_2^-$ was assayed on cells that were pretreated with sepiapterin, the effects of GA and l-NAME (Fig. 1C) were essentially the same as that obtained from non-sepiapterin-treated cells (Fig. 1B). On the basis that l-NAME is a well recognized substrate analog inhibitor of eNOS and an ineffective agent for blocking relaxation in large vessels from eNOS knock-out mice (12), these data indicate that GA uncouples eNOS to generate $O_2^-$. In addition, the data from the sepiapterin-treated cultures suggest that GA does not uncouple eNOS activity by limiting BH4.

The above observations demonstrate that GA increases the release of $O_2^-$ by shifting the balance of NO and $O_2^-$ production by eNOS in cultured endothelial cells. To determine whether GA induces similar shifts in oxidant balance in vascular endothelial cells in situ, the effects of GA on $O_2^-$ generation by canine carotid arteries were measured. The endothelium of untreated carotid arteries exhibit little to no staining with hydroethidine (Fig. 2, left). Deepier optical sectioning of the arteries was unable to detect ethidine staining in smooth muscle cells of the vessel wall (data not shown). These data suggest that GA increases vascular $O_2^-$ generation by an endothelium-dependent mechanism.

Serine phosphorylation of eNOS at Ser-1177 (human) and Ser-1179 (bovine) has been shown to correlate directly with increased NO production (13). Treatment of BCEC cultures with A23187 increased phospho-eNOS(Ser-1177) levels compared with the levels seen in V-treated cultures (Fig. 3A, top panel). Pretreatment with GA alone increased phospho-eNOS levels (>2.5-fold) compared with V-treated cultures. A23187 stimulation of GA-treated cultures also increased phospho-eNOS. These data indicate that GA increases serine phosphorylation of eNOS, a marker of increased eNOS activity (2, 14). The observation that A23187 simultaneously increases l-NAME-inhibitable $O_2^-$ generation and eNOS phosphorylation in GA-treated cultures suggests that when Hsp90 is bound to eNOS in the ADP conformation, enzyme activity is uncoupled. Although the phosphorylation of eNOS stimulated by growth factors or shear stress has been shown to correlate directly with NO production (13), if phospho-eNOS levels are detected in the absence of NO, perhaps the phosphorylation of eNOS reflects uncoupled eNOS activity and enhanced electron flux in order to generate $O_2^-$ as was shown previously (14).

Hsp90 serves many functions within cells (see Ref. 15 for review). It assists in protein folding by first binding and then changing the conformation of the Hsp90 bound to the client proteins. In this respect, GA has been a useful tool for distinguishing between the steps of association and conforma-
in protecting enzyme function (16). To begin to understand how each step plays a role in preserving eNOS function co-precipitation studies of eNOS were performed on V-treated, V-treated + A23187, GA-treated, and GA-treated + A23187-stimulated test groups. None of these treatments affected eNOS levels (Fig. 3B, upper panel). Independently GA and A23187 slightly increased the levels of Hsp90 that co-precipitated with eNOS (Fig. 3B, lower panel, lanes 2 and 3, respectively). In combination however, they markedly increased the amount of Hsp90 that could be co-immunoprecipitated with eNOS (Fig. 3B, lower panel, lane 4). These observations are consistent with the report showing GA increases Hsp90 binding to heat-denatured luciferase but not necessarily enzyme activity (16). On the basis that GA inhibits conformational changes in Hsp90 and changes eNOS product formation from NO to O₂ we conclude that simply binding Hsp90 to eNOS without the ability to change conformation is insufficient to preserve eNOS-dependent NO generation. These data indicate that Hsp90 binding and conformational activity are important for limiting eNOS-dependent O₂ generation.

**DISCUSSION**

This study shows that altering the interaction of Hsp90 with eNOS uncouples enzyme activity resulting in increased O₂ generation. Impaired nitrite production and increased L-NAME-inhibitable O₂ generation by A23187-stimulated, GA-treated cultures indicate that Hsp90 modulates eNOS-dependent NO and O₂ generation. In situ studies reveal that GA increases vascular O₂ generation in the endothelium but not smooth muscle cells. Such specificity is consistent with the observation that Hsp90 and eNOS are found to co-localize in the endothelium, and that their interaction is critical for mediating vasorelaxation and vascular tone (1, 2, 4). Increased phospho-eNOS(Ser-1179) levels in A23187-stimulated cultures pretreated with GA suggest that eNOS-dependent O₂ generation and NO production may share common signaling mechanisms. The marked increase in the amount of Hsp90 that can be co-precipitated with eNOS in A23187-stimulated GA-pretreated cultures indicates that the ability of Hsp90 to change conformation is essential to the mechanisms by which Hsp90 increases NO and limits O₂ production by eNOS. To our knowledge this is the first demonstration where the ATP/ADP state of Hsp90 influences different enzyme activities of a single protein.

By measuring the effects of GA on nitrite and O₂ production in the absence and presence of well recognized eNOS inhibitors we were able to determine how GA altered eNOS function. What we observed was that when GA decreased A23187-stimulated nitrite production it also increased A23187-stimulated O₂ generation by a mechanism that could be inhibited by L-NAME, which is now interpreted as an eNOS-dependent mechanism. In the present study, the finding that A23187 markedly increases phospho-eNOS in GA-treated cultures is entirely consistent with the idea that the flow of electrons through eNOS was increased (14). However when pretreating with GA, the increase in electron flow resulted in increased O₂ generation by an activated eNOS.

Superoxide anion can be generated by eNOS at either the NADPH reductase domain or the arginine oxidase domain (8, 17–20). Xenobiotics such as paraquat, lucigenin, and adriamycin as well as excess FAD and FMN increase O₂ generation via redox-cycling with the NADPH reductase domain of eNOS (8, 17–20). “Uncoupled” eNOS activity develops when eNOS fails to “couple” activated oxygen to arginine metabolism. When this happens, the activated oxygen is released from the heme site as...
O$_2^-$ (21). eNOS-dependent O$_2^-$ generation from the arginine oxygenase domain has been observed under conditions of limited L-arginine availability, oxidation or depletion of tetrahydrobiopterin, and activation of protein kinase C (6, 22, 23). To determine whether GA increased eNOS-dependent O$_2^-$ generation by a BH4-dependent mechanism BCEC cultures were incubated with sepiapterin, which is rapidly converted to BH4 by the salvage pathway (24). Not surprisingly, GA increased A23187-stimulated O$_2^-$ generation and more importantly L-NAME decreased the release of O$_2^-$ to the same extent as they did in the non-sepiapterin-treated cultures. These data suggest that mechanisms targeting Hsp90 activity may be distinct from the mechanisms by which a loss in BH4 increases eNOS-dependent O$_2^-$ generation (25, 26).

On the basis that GA is a specific inhibitor of Hsp90 activity and BH4 supplementation did not inhibit the effects of GA on eNOS-dependent O$_2^-$ generation, we hypothesize that the way Hsp90 interacts with eNOS plays an important role in mediating the balance of -NO and O$_2^-$ generation by eNOS. What these data seem to indicate is that when high levels of phospho-eNOS are detected in cells or tissues that generate low concentrations of -NO, citrulline or cGMP eNOS activity may be uncoupled. On the basis of known stoichiometry for arginine metabolism by NOS (27) it is clear that for each -NO made by coupled activity, two molecules of O$_2^-$ can be generated by uncoupled activity (21). The ability of an enzyme to generate vasoactive radicals with opposing physiological properties may actually be an advantage in that mechanisms mediating vasorelaxation and vasoconstriction could be integrated into a single control point. The importance of uncoupled eNOS activity to vascular physiology remains unclear at this time. Future studies aimed at understanding how vasoactive agents alter Hsp90 activity in relation to eNOS generation of -NO and O$_2^-$ are required to determine the extent to which such a mechanism plays a role in vasorelaxation and vascular tone (1).

In conclusion, Hsp90 modulates eNOS product formation. When Hsp90 is bound to eNOS and can change conformation, eNOS generates -NO upon stimulation. When Hsp90 is bound to eNOS and conformational changes are restricted or impaired, eNOS generates O$_2^-$ upon stimulation. Thus, Hsp90 modulates eNOS production formation may play an important role in vascular physiology as well as atherosclerosis, hypertension, and diabetes. Such observations begin to explain some of the hypertensive and anti-angiogenic effects of inhibiting Hsp90 activity.

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