Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension

VIJAY SHAH,1,2,3,4 REINER WIEST,2,4 GUILLERMO GARCIA-CARDENA,5 GREG CADELINA,2,4 ROBERTO J. GROSZMANN,1,2,4 AND WILLIAM C. SESSA5

Departments of 1Medicine and 3Pharmacology and 2Hepatic Hemodynamic Laboratory, Yale University School of Medicine, New Haven CT; 4Connecticut Veterans Affairs Healthcare Center, West Haven, Connecticut 06536; and 5Gastrointestinal Research Unit, Mayo Clinic, Rochester, Minnesota 55903

Shah, Vijay, Reiner West, Guillermo Garcia-Cardena, Greg Cadelina, Robert J. Groszmann, and William C. Sessa. Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G463-G468, 1999.—The molecular chaperone, heat shock protein 90 (Hsp90), acts as an intermediate in the signaling cascades leading to activation of endothelial nitric oxide synthase (eNOS). In this study, we examine the participation of this pathway in nitric oxide (NO)-dependent vasodilation in the rat mesentery in vitro. In normal animals, immunoprecipitation of eNOS from intact mesentery coimmunoprecipitates Hsp90 and, additionally, both eNOS and Hsp90 colocalize to the endothelial lining of mesenteric vessels. In the perfused mesenteric vasculature of normal animals, geldanamycin (GA), a specific inhibitor of Hsp90 signaling, attenuates ACh-dependent vasodilation but does not affect vasodilation in response to sodium nitroprusside. Next, studies were performed in animals with experimental portal hypertension induced by portal vein ligation (PVL). In PVL animals, NO synthase catalytic activity is markedly enhanced in mesenteric tissue and the perfused mesentery is hyporesponsive to the vasoconstrictor methoxamine (MTX). GA significantly potentiates MTX-induced vasoconstriction after PVL, thereby partially reversing the hyporeactivity to this agent exhibited in the mesenteric vasculature after PVL. These studies suggest that Hsp90 can act as a signaling mediator of NO-dependent responses in the mesenteric circulation and indicate that the excessive NO production observed in portal hypertension is mediated in part through Hsp90 signaling.

Vessel homeostasis is maintained by a balance of vasoactive substances and hemodynamic forces, including shear stress, which mediates vascular responses in part through modulation of endothelial nitric oxide synthase (eNOS). eNOS-derived nitric oxide (NO) production is regulated through distinct posttranslational mechanisms, one being the interaction of NO with regulatory proteins (8, 10, 12, 13). In support of this concept, we have recently demonstrated that the interaction of eNOS with heat-shock protein 90 (Hsp90) facilitates NO production in response to mechanical stimuli, growth factors, and G protein-coupled signaling pathways and, additionally, that specific inhibition of Hsp90 signaling with the ansamycin antibiotic geldanamycin (GA) inhibits NO production (8).

Vascular tone in the mesenteric circulation contributes to regulation of portal pressure. In clinical and experimental portal hypertension, mesenteric vasodilation and increased splanchnic blood flow elevates portal venous inflow, thereby contributing to increased portal pressure (17). Pressure and resistance changes in the perfused mesenteric vasculature occur, in part, through NO-dependent mechanisms, and in experimental portal hypertension this vascular bed demonstrates a hyporeactivity to vasoconstrictors such as methoxamine (MTX), due in large part to excessive endothelium-derived NO production (19, 20). The recently demonstrated importance of Hsp90 as an intermediate in the signaling cascades leading to activation of eNOS (8) suggests the possibility of a contributory role of this pathway in NO-dependent mesenteric vascular responses and excessive NO production in experimental portal hypertension.

Therefore, the goals of this study were to 1) examine eNOS and Hsp90 coassociation and localization in rat mesenteric microvasculature, 2) determine whether GA inhibits NO-dependent mesenteric vasoconstriction, and 3) test whether GA reverses the hyporeactivity to vasoconstrictors detected in the mesenteric vasculature of portal hypertensive animals.

MATERIALS AND METHODS

Animals and reagents. Male Sprague-Dawley rats (Harlan Sprague Dawley Laboratories, Indianapolis, IN) weighing 300–375 g were used for these experiments. Animal experiments and tissue harvesting were performed in accordance with the animal care guidelines of the institution. GA (GIBCO, Grand Island, NY) was dissolved in DMSO, and the final concentration of DMSO used in experiments was <0.006%. Solutions of MTX, ACh, and sodium nitroprusside (SNP), all obtained from Sigma Chemical (St. Louis, MO), were dissolved in distilled water and prepared daily. The equivalent volume of Krebs solution or DMSO required for solution of the compound was used as control vehicle in all experiments.

Immunohistochemistry. Mesenteric tissue was perfusion fixed in situ with 4% paraformaldehyde, postfixed in sucrose, and embedded in OCT (16). Frozen tissue sections were incubated overnight with eNOS monoclonal antibody (MAb; Transduction Laboratories, Lexington, KY) or Hsp90 MAb (Stressgene Biotechnologies, Victoria, BC, Canada), and secondary staining was performed with horse anti-mouse from Vector Laboratories (Burlingame, CA) for 30 min. After they were labeled with streptavidin-peroxidase from Boehringer-Mannheim (Indianapolis, IN), slides were developed with

0193-1857/99 $5.00 Copyright © 1999 the American Physiological Society
aminoethyl carbazole. Negative control slides were incubated with appropriate serum substituted for primary antibody.

Induction of portal hypertension. Surgical constriction of the portal vein (portal vein ligation, PVL) was performed as previously described (6, 19). In brief, rats were anesthetized, and, after laparotomy, the portal vein was dissected and isolated. A 20-gauge blunt-tipped needle was placed alongside the portal vein, and a ligature (3-0 suture) was tied around the needle and the vein. The needle was immediately removed, yielding a calibrated stenosis of the portal vein. Sham surgery was performed in a manner identical to the protocol for PVL except no ligature was tied around the portal vein.

Immunoprecipitation and Western blotting. Mesenteric tissue was harvested by dissecting and removing the highly vascular tissue situated between the mesenteric lymph nodes and small intestine. The tissue was washed in PBS and homogenized in a lysis buffer [50 mM Tris-HCl, 0.1 mM EGTA, 0.1 mM EDTA, 100 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% (vol/vol) NP-40, 0.1% SDS, and 0.1% deoxycholic acid; pH 7.5] as previously described (18). Protein quantification of tissue samples was performed using the Lowry assay. In experiments examining the levels of eNOS, inducible nitric oxide synthase (iNOS; Affinity Bioreagents, Golden, CO), and Hsp90 protein in mesenteric tissue, 100 µg of protein were used for electrophoresis. For eNOS immunoprecipitation studies, 1,500 µg of detergent-soluble protein was incubated with excess eNOS MAb overnight after samples were precleared with Pansorbin (Calbiochem, La Jolla, CA). Immune complexes were precipitated by the addition of protein A-Sepharose (Pharmacia, Piscataway, N J). Protein samples were boiled in Laemmli loading buffer and separated by SDS-PAGE on a 7.5% acrylamide gel, and proteins were transferred to a nitrocellulose membrane. The membranes were washed in Tris-buffered saline with 0.1% Tween, blocked in 5% milk, and incubated with either Hsp90 MAb or eNOS MAb.

In vitro mesenteric perfusion studies. In vitro mesenteric perfusion studies were performed 14 days after PVL or after sham surgery, using a modification of the method originally described by McGregor and previously described in our laboratory (19–21). In brief, the superior mesenteric artery (SMA) was cannulated with a PE-60 catheter, and blood was removed by perfusion with 15 ml of warmed Krebs solution. The gut was dissected at the mesenteric border, and the SMA with its adjoining mesenteric tissue was placed in a 37°C water-jacketed container. The preparation was continuously perfused in a nonrecirculating system at a fixed rate of 4 ml/min throughout the course of the experiment with Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, 25 NaHCO3, 0.026 EDTA, and 11 glucose, pH 7.4; 95% O2-5% CO2). Perfusion pressure was continuously monitored using a strain gauge transducer (Statham, Oxnard, CA) on a side-arm proximal to the perfusing cannula. The initial preparation was allowed to stabilize for 30 min, after which vehicle was infused for 15 min. Immediately after infusion of vehicle, concentration-response curves were examined in response to MTX infusion (30 and 100 µM). When a stable baseline was maintained in response to 100 µM MTX, responses to ACh boluses (0.1 ml) were examined (1 and 10 µg). All compounds were allowed to wash out, and the next curve after which GA was infused (3 µg/ml) for 15 min. Responses to MTX and ACh were repeated as described above. Additional experiments were performed after endothelial denudation. Denudation was achieved by combined treatment with cholic acid and distilled water as previously described (1). In brief, after cannulation of the SMA and gentle flushing with 10 ml of warmed Krebs solution to eliminate blood, the mesentery was perfused with 1.5 ml of 0.5% cholic acid for 10 s followed by 15 ml of warmed Krebs solution. The preparation was then transferred to a 37°C water-jacketed container and perfused with warmed, oxygenated Krebs solution for 10 min. After the mesenteric vasculature was relaxed, warmed distilled water was perfused for 10 min. After a 45-min stabilization period, vehicle or, alternatively, GA was perfused for 15 min. Immediately after pretreatment with vehicle or GA, an infusion of 100 µM MTX was begun. When a stable baseline was maintained in response to 100 µM MTX, concentration-response curves to 0.1-mi SNP boluses (0.001–10 µg) were examined. Additional experiments were also performed in sham and PVL mesenteric vessels in response to GA preinfusion using MTX doses that allowed equivalent levels of constriction in response to preinfusion of vehicle. Equivalent constriction was achieved with 10 and 30 µM MTX in sham and PVL mesentery, respectively.

NOS activity assays. The conversion of 3H-labeled L-arginine to 3H-labeled L-citrulline was used to determine NOS activity (9). Briefly, mesenteric tissue, harvested as described above, was homogenized in a lysis buffer identical to that used for immunoprecipitation studies. Samples were incubated with a buffer containing 1 mM NADPH, 3 mM tetrahydrobiopterin, 100 nM calmodulin, 2.5 mM CaCl2, 50 mM L-valine, 10 µM L-arginine, and L-[3H]arginine (0.2 µCi) at 37°C. To determine NOS activity, duplicate samples were incubated in the presence and absence of Nω-nitro-L-arginine methyl ester (LNAME; 1 mM) or vehicle. After incubation for 20 min, the reaction was terminated by the addition of 1 ml of cold stop buffer (20 mM HEPES, 2 mM EDTA, and 2 mM EGTA, pH 5.5), and the reaction mix was passed over a column containing Dowex AG 50WX-8 resin into a vial and analyzed using a liquid scintillation counter. Radiolabeled counts per minute of L-citrulline generation were measured.

Statistics. All data are expressed as means ± SE. Statistical analysis was performed using paired and unpaired Student’s t-tests as well as ANOVA where appropriate.

RESULTS

Coassociation and localization of eNOS and Hsp90 in normal mesenteric tissue. To determine the importance of Hsp90 signaling in the mesenteric vasculature, we first examined the expression, association, and cellular localization of eNOS and Hsp90 in the mesentery. Both eNOS and Hsp90 are highly expressed in mesenteric tissue as demonstrated by Western blotting (Fig. 1A, left). In addition, immunoprecipitation of eNOS coprecipitates Hsp90 from mesenteric tissue (Fig. 1A, right), consistent with the interaction in cultured endothelial cells (8). As depicted in Fig. 1B, immunoperoxidase staining using specific MAbs demonstrates that both eNOS (left) and Hsp90 (right) are present in the endothelial lining of mesenteric vessels. Although eNOS staining is visualized exclusively within the vascular endothelium, immunostaining for Hsp90 is prominently observed in vascular smooth muscle cells of the media as well.

GA attenuates ACh-dependent vasorelaxation of the normal isolated perfused rat mesentery. To determine the physiological significance of Hsp90 coassociation with eNOS in the mesenteric vasculature, we performed in vitro mesenteric perfusion studies with GA, a pharmacological inhibitor of Hsp90 signaling. In this preparation, ACh induces vasorelaxation in a dose-dependent manner after preconstriction of the circulation with MTX. As depicted in Fig. 2A, mesenteric
vasorelaxation in response to ACh (1 and 10 µg) is significantly attenuated after preinfusion of GA (3 µg/ml) compared with preinfusion of vehicle \((P, 0.05, \text{vehicle vs. GA; } n = 5)\). Next, studies were performed to examine if GA affects the direct vasodilatory actions of an NO donor. Pretreatment with GA does not affect vasodilation in response to SNP \((n = 6)\) (Fig. 2B), indicating that the effects of GA are dependent on the endothelium and that GA does not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation.

NOS catalytic activity, protein levels, and mesenteric vascular responses in portal hypertensive animals. To examine NOS catalytic activity in mesenteric tissue from PVL and sham animals, assays were performed to measure the conversion of \(^3\)H-labeled l-arginine to \(^3\)H-labeled l-citrulline. Equal amounts of sham and PVL mesenteric tissue were homogenized and incubated with \(^3\)H-labeled l-arginine and all essential cofactors necessary for optimal NOS activity in the presence and absence of l-NAME. NOS activity was significantly increased in mesenteric tissue from PVL animals compared with sham animals studied in parallel.

![Fig. 1. Coassociation and localization of endothelial nitric oxide synthase (eNOS) and heat-shock protein 90 (Hsp90) in the mesenteric endothelium. A: eNOS was immunoprecipitated from detergent-soluble protein lysates prepared from intact mesenteric tissue harvested as described in MATERIALS AND METHODS and prepared for gel electrophoresis or, alternatively, protein samples were directly prepared for gel electrophoresis. eNOS and Hsp90 are both expressed abundantly in mesenteric tissue (left), and immunoprecipitation (IP) of eNOS coprecipitates Hsp90 under basal conditions (right), wb, Western blot. B: mesenteric tissue was fixed and prepared for immunohistochemistry as described in MATERIALS AND METHODS. Immunoperoxidase staining for both eNOS (left) and Hsp90 (right) is visualized within the endothelial lining of mesenteric vessels (×400). Although eNOS staining occurs exclusively within vascular endothelium, immunostaining for Hsp90 is also demonstrated in mesenteric smooth muscle cells.](http://ajpgi.physiology.org/)

![Fig. 2. Inhibition of Hsp90 signaling attenuates ACh- but not sodium nitroprusside (SNP)-induced vasorelaxation. Mesenteric tissue was perfused in vitro after in situ cannulation of the superior mesenteric artery (SMA). A: vascular responses to ACh in methoxamine (MTX)-preconstricted vessels were examined after infusion of vehicle or geldanamycin (GA). Preinfusion of GA attenuates vasorelaxation in response to ACh \((n = 5; *P < 0.05, \text{vehicle vs. GA at ACh doses of } 1 \text{ and } 10 \mu g)\). B: after cannulation of the SMA, endothelium was denuded with cholic acid and distilled water as described in MATERIALS AND METHODS. Vascular responses to SNP in MTX-preconstricted vessels were examined after infusion of vehicle or GA. GA has no significant effect on vasorelaxations in response to SNP \((n = 6; \text{SNP doses of } 0.001–10 \mu g)\).](http://ajpgi.physiology.org/)
Hsp90 protein levels were also similar between the two groups (Fig. 3D). Portal hypertensive animals demonstrated a significant reduction in baseline perfusion pressure [sham (n = 5 animals): 20.2 ± 0.74 mmHg; PVL (n = 8 animals): 18.6 ± 0.60 mmHg; P < 0.05, sham vs. PVL] as well as a concentration-dependent hyporeactivity to MTX (Fig. 4A) (P < 0.05, sham vs. PVL at MTX concentrations of 30 and 100 µM), as previously demonstrated (19). GA potentiates mesenteric vascular responses to MTX in portal hypertensive animals. On the basis of the importance of Hsp90-eNOS interactions in the normal mesenteric circulation and the excessive NOS catalytic activity demonstrated in the mesenteric vasculature of PVL animals, we wondered whether Hsp90 might be of importance in influencing the hyporeactivity to MTX observed in portal hypertension. As depicted in Fig. 4A, GA significantly potentiates the increase in perfusion pressure in response to 30 µM MTX in both sham and PVL animals (P < 0.05, GA vs. vehicle; n = 5 sham and n = 8 PVL). In response to 100 µM MTX, GA significantly potentiates the increase in perfusion pressure in portal hypertensive animals (P < 0.05, GA vs. vehicle) but not in sham animals. In addition, the percent increase in perfusion pressure in response to both doses of MTX after GA pretreatment is significantly greater in portal hypertensive animals compared with sham animals, suggesting a preferential effect of GA in portal hypertensive animals. (P < 0.05, sham vs. PVL) (Fig. 4B). To exclude the possibility that the preferential effects of GA in the perfused mesentery from PVL compared with sham-ligated rats occurred due to a reduced pressor response to MTX in PVL animals, the effects of GA were examined after equivalent perfusion pressures were achieved in both sham and PVL mesenteric vasculatures. MTX (10 and 30 µM) increased perfusion pressure by 58.7 ± 7.5 and 55.3 ± 5.2 mmHg in sham and PVL rats, respectively. Under these matched perfusion pressures, GA increased perfusion pressure to a greater extent in portal hypertensive animals compared with sham animals (P < 0.05, n = 10 sham and 12 PVL rats, Fig. 4C). These data suggest that enhanced activation of Hsp90-mediated pathways leading to NO production occurred in PVL animals.

**DISCUSSION**

Hsp90 acts as an intermediate in the signaling cascades leading to activation of eNOS (8). In this study, we examined the role of this protein interaction in mediating vasoreactive responses in the mesenteric vasculature, a site in which vascular resistance contributes to portal pressure regulation. The novel findings of this study include the interaction between Hsp90 and eNOS in resistance vessels and the participation of this interaction in regulating vasomotor function in resistance vessels based on inhibition of Hsp90 signaling with GA. Moreover, we show that, after PVL, enhanced Hsp90 signaling contributes to NO-dependent vascular hyporeactivity, thus providing a mechanism linking protein-protein interactions with the vascular manifestations observed in portal hypertension.

The molecular chaperone, Hsp90, facilitates the folding and stabilization of cellular proteins, thereby promoting specific signaling pathways (14). GA is used as a specific reagent to probe the importance of Hsp90 in cellular pathways, since it directly binds to the amino-terminal ATP binding domain of Hsp90 (15, 22) and specifically labels Hsp90 in cellular extracts (23). Activation of protein kinases such as v-Src and Raf-1 and facilitation of steroid hormone binding with nuclear receptors are signaling events dependent on Hsp90 and blocked by GA (2, 7, 14). The present study demonstrates that GA also blocks vascular responses mediated by eNOS, albeit upstream from the site of action of traditional NO biosynthesis inhibitors such as arginine-substituted analogs, without influencing the direct

---

**Fig. 3.** NOS catalytic activity is enhanced in portal hypertensive mesenteric microvasculature 14 days after portal vein ligation (PVL). A: NOS activity was assessed in homogenized mesenteric tissue from sham (n = 5) and PVL (n = 5) rats studied in parallel, as described in MATERIALS AND METHODS. NOS activity is significantly enhanced in mesenteric tissue from PVL animals compared with sham animals (P < 0.05, paired t-test). B–D: detergent-soluble protein lysates were prepared from intact mesenteric tissue from sham and PVL rats, prepared for gel electrophoresis, and Western blotted using eNOS and inducible nitric oxide synthase (iNOS) and Hsp90-specific antibodies. Rat sinusoidal endothelial cells (SEC) stimulated with lipopolysaccharide for 18 h (0.5 µg/ml) were used as a positive control for both eNOS and iNOS. B: representative blot demonstrating that eNOS protein levels are similar or slightly increased in 2 PVL animals compared with 2 sham animals (n = 7 for each group). C: representative blot demonstrating that iNOS induction is not detected in 2 sham or 2 PVL animals (n = 4 for each group). D: representative Western blot demonstrating that Hsp90 levels are not increased in PVL animals compared with sham animals 14 days after PVL (this is representative of blots from 4 rats in each group).
vasodilatory effects of SNP. These data, in conjunction with the above evidence for the specificity of GA, suggest that Hsp90 participates as a regulator of endothelial cell signal transduction leading to eNOS activation and vasorelaxation.

The hemodynamic abnormalities observed in the mesenteric circulation after PVL are mediated in large part by excess production of NO. Endothelial denudation of the mesenteric vasculature normalizes vascular responses after PVL, and immunostaining in conduit vessels demonstrates the presence of eNOS but not iNOS (1). In addition, eNOS activity is increased in conduit vessels harvested from animals 14 days after PVL (3, 4). The present study indicates that NOS catalytic activity in the PVL rat is increased in the mesenteric macro- and microcirculation distal to the SMA, where vascular resistance and portal venous inflow are determined. Our findings that inhibition of Hsp90 signaling preferentially increases responsiveness to vasoconstrictors in portal hypertension are particularly provocative in light of recent studies demonstrating enhanced G protein-induced relaxation and functional activity in vessels from portal hypertensive animals (5, 11). These studies, in conjunction with our recent demonstration that activation of eNOS through G protein-coupled receptors is facilitated through Hsp90 signaling (8), suggest that NOS activation in the portal hypertensive vasculature may be linked to a G protein-coupled stimulus that activates eNOS through a signaling pathway dependent on Hsp90. However, the inability of GA to completely reverse the hyporesponsiveness to MTX in portal hypertensive mesentery suggests that other regulatory pathways for NOS activation in portal hypertension are also involved. In addition, it is pos-

Fig. 4. A: GA potentiates MTX-induced vasoconstriction in the portal hypertensive mesenteric vasculature. To examine whether inhibition of Hsp90 signaling might reverse the hyporeactivity to MTX, mesenteric perfusion studies were performed 14 days after PVL or sham surgery. A: in sham rats, preinfusion of GA potentiates the vasoconstriction induced by 30 µM MTX as demonstrated by an increase in mesenteric perfusion pressure but not that of 100 µM MTX (*P < 0.05, GA vs. vehicle at 30 µM MTX; n = 5). In PVL rats, preinfusion of GA potentiates MTX (30 and 100 µM)-induced vasoconstriction in the mesenteric vasculature (*P < 0.05, GA vs. vehicle; n = 8). B: expression of the data in A; %change in perfusion pressure demonstrates the marked potentiation of MTX-induced increase in perfusion pressure in PVL rats after GA pretreatment compared with sham rats after GA pretreatment (*P < 0.05). C: after equivalent degrees of vasoconstriction were obtained in both sham and PVL mesenteric vasculatures (see RESULTS), GA pretreatment significantly potentiates the increase in perfusion pressure in response to MTX in PVL compared with sham rats (*P < 0.05; n = 10 sham and 12 PVL rats).
sible that very low levels of iNOS induction not detected by Western blot and/or small increases in eNOS protein might also contribute to enhanced NOS activity in portal hypertensive vessels.

In summary, this study provides strong evidence for a functional link between eNOS activation and Hsp90 signaling. This interaction likely serves to mediate NO-dependent responses in the perfused mesenteric vasculature. In addition, this study implicates a contributory role for Hsp90 signaling in the excessive NOS activity and NO-dependent hyporeactivity observed in portal hypertension.

GA was a generous gift from Dr. Len Necker and the National Cancer Institute. We acknowledge the technical assistance of Ken Choo and Radu Daniel Rudic.

This work was supported by grants from the National Institutes of Health (HL-57665 and HL-51948 to W. C. Sessa and DK-02529 to V. Shah), Veterans Affairs Merit Review (to R. J., Grozmann, Glaxo Institute of Digestive Health (to V. Shah), and a Liver Center Pilot Project Grant (to V. Shah). W. C. Sessa is an Established Investigator of the American Heart Association. V. Shah is the recipient of a Lucille Markey Physician Scientist Award. The contributions of the first two authors are equal.

Present address of G. Garcia-Cardena: Vascular Research Division, Harvard Medical School, 221 Longwood Ave. LMRC-4, Boston, MA 02115-5817.

Address for reprint requests and other correspondence: W. C. Sessa, Boyer Center for Molecular Medicine, Rm. 436B, Yale Univ. School of Medicine, 295 Congress Ave., New Haven, CT 06536-0812 (E-mail: william.sessa@yale.edu).

Received 25 November 1998; accepted in final form 7 May 1999.

REFERENCES


