Heat shock protein 90 is responsible for hyperdynamic circulation in portal hypertensive rats

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AIM: To examine the participation of HSP90 in portal hypertensive rat mesentery in vitro.

METHODS: Immunohistochemistry and Western blot were used to examine the expression of HSP90 in mesenteric vasculature. HSP90 mRNA was detected by RT-PCR, and the role of HSP90 in hyperdynamic circulation was examined by in vitro mesenteric perfusion studies.

RESULTS: HSP90 was overexpressed in endothelium of mesenteric vasculature in animals with experimental portal hypertension induced by partial portal vein ligation (PVL) compared with normal animals. Geldanamycin (GA), a special inhibitor of HSP90 signaling, attenuated ACh-dependent vasodilation but did not affect vasodilation in response to sodium nitroprusside in normal rats. In PVL animals, the perfused mesentery was hyporesponsive to vasoconstrictor after PVL. GA significantly potentiated methoxamine-induced vasoconstrictor after PVL.

CONCLUSION: HSP90 plays a key role in NO-dependent hyperdynamic circulation in portal hypertension and provides a novel method for future treatment of portal hypertension.

Introduction

Cirrhosis of the liver, which usually develops as a long-term consequence of viral hepatitis or alcohol abuse, is a major cause of morbidity and mortality worldwide. The principal pathophysiological feature of cirrhosis is an increase in portal pressure initiated by an increase in outflow resistance to the portal circulation. However, advanced cirrhosis was also associated with mesenteric arteriolar vasodilation[1], which contributes to portal hypertension and variceal hemorrhage by increasing portal inflow. It has been well established that portal hypertension was not a purely mechanical phenomenon[2]. There have been a large body of experimental evidences that demonstrated increased synthesis of nitric oxide (NO) in animal models of cirrhosis. Aortic cAMP, a surrogate marker of NO synthesis, was significantly elevated in CCL-induced cirrhosis rats compared to controls and the highest levels were observed in those with ascites[3]. Similar evidences also existed for elevated NOS activity in animal models of portal hypertension induced by partial vein ligation[4]. NO and endothelin-1-dependent increases in intrahepatic resistance in conjunction with vasodilation of splanchnic arteries raised portal pressure and flow, thereby contributing to the vascular component of portal hypertension[5]. Indeed, the importance of splanchnic vasodilation in this process was highlighted by clinical utility of nonselective beta-blockers and octreotide, both of which could reduce splanchnic vasodilation and portal venous inflow[6]. However, cirrhotic vasculature was highly resistant to conventional vasopressors[7], and attempts to correct the hyperdynamic circulation in cirrhosis by antagonism of putative endogenous vasodilator mediators have been unsuccessful so far[8,9]. Pressure and resistance changes in perfused mesenteric vasculature occurred, in part through NO-dependent mechanisms, and in experimental portal hypertension this vascular bed demonstrated a hyporeactivity to vasoconstrictors such as methoxamine (MTX), mainly due to excessive endothelium-derived NO production[10,11]. NO is derived from L-arginine by the enzymatic action of NOS. Two isoforms of NOS have been shown to exist in the vasculature, one is a constitutively expressed and calcium-caldemulin-dependent isoform (eNOS)[12], the other is an inducible and calcium-independent isoform (iNOS)[13,14]. Indirect evidences for eNOS as the main source of elevated NO from studies showed that endothelial denudation could normalize vascular levels of NO[14] and reverses the hyporeactivity to vasoconstrictors[15,16] in cirrhotic rats. Several studies now suggested that eNOS was the major enzymatic source of NO overproduction[17-20] in vascular endothelium in cirrhosis. However, the molecular mechanism is still unknown. 90 kD heat shock protein (HSP90) has been found to be a molecular chaperon, and a constitutive homodimer, its main intersubunit binding site for geldanamycin (GA), a representative of ansamycin drugs, which specially targets HSP90[21]. HSP90 mediates the conformational regulation of a wide range of client proteins involved in signal transduction, cell proliferation and apoptosis. The recently demonstrated importance of HSP90 as an intermediate in the signaling cascades leading to activation of eNOS[22] suggested the possibility of a contributory role of this pathway in NO-dependent mesenteric vascular responses and excessive NO production in experimental portal hypertension.

The goals of this study were to examine HSP90 expression and localization in rat mesenteric microvasculature, to determine whether GA inhibited mesenteric vasodilation, and to test whether GA reversed the hyporeactivity to vasoconstrictors detected in mesenteric vasculature of portal hypertensive animals.
MATERIALS AND METHODS

Animals and reagents
Male Sprague-Dawley rats (Tongji Hospital Laboratories) weighing 250-300 g were used for experiments. Animal experiments and tissue harvesting were performed in accordance with the animal care guidelines of the institution. GA was dissolved in DMSO, and the final concentration of DMSO used in experiments was <0.006%. MTX, ACh, and sodium nitroprusside (SNP) obtained from Sigma Chemical, were dissolved in distilled water and prepared daily.

Induction of portal hypertension
Prehepatic portal hypertension was provoked by partial portal vein ligation (PVL) as previously described[13]. In brief, rats were anesthetized, and after laparotomy, the portal vein was isolated and a stenosis created by placing a single ligature of 3-0 silk around both the portal vein and a 20-gauge blunted tipped needle. The needle was then removed from the ligature, creating a calibrated constriction of the portal vein. In sham-operated (SO) rats, the portal vein was isolated but not ligated. Studies were performed in 24 hours fasted rats 21 days after PVL.

Immunohistochemistry
Mesenteric tissue was perfusion fixed in situ with 4% paraformaldehyde, postfixed in sucrose, and embedded in OCT[23]. Frozen tissue sections were incubated overnight with HSP90 MAb, and a secondary incubation was performed with horse anti-mouse IgG for 30 min. Sections were developed with amino ethylcarbazole. Negative controls were incubated with appropriate serum substituted for the primary antibody.

RNA isolation and reverse transcriptase and polymerase chain reaction (RT-PCR)
Mesenteric tissue was harvested by dissecting and removing the highly vascular tissue situated between the mesenteric lymph nodes and small intestine. Mesenteric specimens from SO and PVL rats were immediately frozen in liquid nitrogen, then homogenized with TriPure isolation reagent to isolate total RNA. The RNA concentration in each sample was determined spectrophotometrically, and the quality of each RNA preparation was documented by visualization of 18S and 28S ribosomal bands after electrophoresis through a 1% agarose gel. Total RNA samples were subjected to reverse transcription with Oligo-dt used as a template-primer. First strand synthesis was carried out for 1 hour at 37°C in a reaction mixture containing 200 U M-MLV, 1X reaction buffer, 0.5 mMol/L dNTP, 10 mMol/L DTT, 20 U RNAsin ribonuclease inhibitor and 0.25 µg oligo-dT. The PCR primer sequences were as follow: HSP90 sense 5’ GTCTGGGTATCGGAAAGCAAG3’, antisense 5’ CTGAGGGTTGGGATGATGTC3’. PCR assays contained 0.5 U Taq DNA polymerase, 0.5 mMol/L of each oligonucleotide primer, 0.2 mMol/L dNTP, 1X reaction buffer, 1.5 mMol/L MgCl2, and a final volume of 50 µl. 25 cycles were performed at 95°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min. PCR products were analyzed by electrophoresis in TAE buffer with ethidium bromide stained with (0.5 mMol/L) 2% agarose gel. Each band was selected and used to measure the number of photons emitted.

Western blotting
Mesenteric sections were weighed and homogenized in 10 volumes of ice-cold 1% NP-40 in 50 mM Tris (pH 8.8) buffer. The homogenates were centrifuged, and the supernatants were transferred into sterile tubes. Fifteen-microgram of protein aliquots of each sample, as determined by the Coomassie blue method, was resolved on 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with BSA before being incubated with primary antibody overnight at 4°C, and rinsed and then incubated for 1 h with peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using the enhanced chemiluminescence system.

In vitro mesenteric perfusion
In vitro mesenteric perfusion was performed 14 days after PVL or after sham surgery, using the method described by McGregor and Sieber with some modifications[26]. In brief, the superior mesenteric artery (SMA) was cannulated with a PE-60 catheter and blood was removed by perfusion with 15 ml of warm 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. Perfusion pressure was continuously monitored using a strain gauge transducer on a sidearm 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 over the next 50 min, 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. The denudation was achieved by combined treatment with cholic acid and distilled water as previously described[27]. In brief, after cannulation of the SMA and gentle flushing with 10 ml of warm Krebs solution to elminate 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-ml 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.

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

RESULTS
Expression and localization of HSP90 in mesenteric vasculature
The expression of HSP90 was significantly enhanced in PVL animals compared with SO and normal animals by Western blot and by RT-PCR (Figures 1A and B). The immunohistochemistry showed that HSP90 staining was not only found in vascular endothelium but also in mesenteric smooth cells (Figure 1C).

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on the endothelium and that GA did not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation. These results showed that HSP90 was an important modulator in vasodilation of mesenteric vasculature.

**DISCUSSION**

This study suggested that HSP90 was overexpressed in the endothelium of mesenteric vasculature in PVL induced animals compared with SO and normal animals. Moreover, in PVL animals, the perfused mesentry was hyporesponsive to the vasoconstrictor methoxamine. GA, a special inhibitor of HSP90 signaling, significantly potentiated methoxamine-induced vasoconstrictor after PVL. It thus indicated a mechanism linking protein-protein interactions with vascular manifestations observed in portal hypertensive hypertension.

HSP90, an ATP-dependent chaperon, facilitated the folding and stabilization of cellular proteins, and played a key role in cellular signal transduction networks. The highly conserved 25 kD NH2-terminal domain of HSP90 was the binding site for ATP and GA, and crystallography has shown that GA occupied the nucleotide-binding cleft within the NH2-terminal domain, which thereby has been used as a specific reagent to probe the importance of HSP90 in cellular pathways. An in vivo requirement for HSP90 has been established for some steroid hormone receptors, several serine/threonine kinases such as pp60/v-src and RAE, and disparate proteins such as NOS and calcineurin. The present study demonstrated that GA also blocked vascular responses mediated by eNOS, without influencing the direct vasodilatory effects of SNP. These data, in conjunction with the above evidences for the specificity of GA, suggested that HSP90 participated in vasodilation of mesentery vasculature as a regulator of endothelial cell signal transduction, leading to eNOS activation and vasorelaxation.

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 eNOS. Two isoforms of NOS have been shown to exist in vascular endothelium but also in mesenteric smooth cells. on the endothelium and that GA did not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation. These results showed that HSP90 was an important modulator in vasodilation of mesenteric vasculature.
inducible and calcium-independent isoform (iNOS)\(^\text{17}\). Several studies suggested that eNOS was the major enzymatic source of NO overproduction\(^\text{17-20}\) in the vascular endothelium in cirrhosis. Our study found that the effects of GA were dependent on the endothelium and that GA did not directly affect soluble guanylate cyclase or other smooth muscle cell machinery required for NO-dependent vasodilation. These studies, in conjunction with the recent demonstration that activation of eNOS was facilitated through HSP90 signaling\(^\text{20}\), suggested that eNOS activation in the portal hypertensive vasculature might be linked to a signaling pathway depending on HSP90. Thus we put forward the hypothesis that HSP90 signaling pathway potentiates the activity of eNOS, which results in NO overproduction and thereby induces hyperdynamic circulation in portal hypertension. However, the inability of GA to completely reverse the hyporesponsiveness to MTX in portal hypertensive rats suggested that other regulatory pathways for NOS activation in portal hypertension were also involved. In addition, it is possible that a small part of NO derived from iNOS might also contribute to hyperdynamic circulation in portal hypertensive vessels.

In summary, this study suggested that HSP90 played a key role in the NO-dependent hyporeactivity observed in portal hypertension. This interaction likely served to mediate NO-dependent responses in the perfused mesenteric vasculature. Taken together, the present study indicates that HSP90 is responsible for hyperdynamic circulation in portal hypertension, and pharmacological inhibition of the signal system may provide a novel target for future treatment of portal hypertensive complications such as ascites and fatal variceal bleeding.

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