Original Article

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Nitric Oxide Synthase Expression in the Splanchnic Hyposensitivity to Glypressin of a Hemorrhage-transfused Rat Model with Portal Hypertension

Hui-Chun Huang[1,4]
Yi-Chou Chen[1]
Sun-Sang Wang[3,4]
Che-Chang Chan[1,4]
Fa-Yauh Lee[1,2,4]
Full-Young Chang[1,4]
Han-Chieh Lin[1,4]
Ming-Chih Hou[1,4]
Rei-Hwa Lu[1]
Shwu-Ling Wu[3]
Shou-Dong Lee[1,4]

Divisions of [1] Gastroenterology and [2] Internal Medicine, Department of Medicine, Taipei Veterans General Hospital, [3] Taipei Municipal Gan-Dau Hospital, and [4] National Yang-Ming University School of Medicine, Taipei, Taiwan, R.O.C.

Key Words
glypressin;
hemorrhage;
nitric oxide;
portal hypertension

Background. Nitric oxide (NO) has been proposed to participate in the vascular hyporesponsiveness to vasopressin and its long-acting analogue glypressin during hemorrhage in portal hypertensive states. This study surveyed the role of NO regarding splanchnic hyporeactivity to glypressin and NO synthases (NOS) expression in different vascular beds in bleeding portal-hypertensive rats.

Methods. Under general anesthesia with ketamine, partially portal vein-ligated male Sprague-Dawley rats without or with bleeding were used to investigate the hemodynamic effects of glypressin (0.07 mg/kg intravenously) and constitutive (cNOS) and inducible NOS (iNOS) mRNA expression over the abdominal aorta and superior mesenteric artery.

Results. Splanchnic hyposensitivity to glypressin was noted in the hemorrhage-transfused rats with enhanced cNOS expression of superior mesenteric artery. No significant differences of cNOS and iNOS expression in abdominal aorta and iNOS in superior mesenteric artery were found between the with-bleeding and without-bleeding groups.

Conclusions. In rats with portal hypertension and acute hemorrhage, cNOS over-expression in superior mesenteric artery may take a part in the splanchnic hyposensitivity to glypressin.

Acute gastric or esophageal variceal bleeding in patients with cirrhosis and/or portal hypertension is an overwhelming condition with high morbidity and mortality. Among various pharmaceutical agents, vasopressin has been universally adopted to cease variceal bleeding via its splanchnic vasoconstrictive activity with subsequently decreased portal venous inflow and pressure.[1,2] However, recent studies indicate that the portal hypotensive effect of vasopressin during acute hemorrhage is less effective than that during stable condition in portal hypertensive model or cirrhotic patients.[3-6] Glypressin, a long-acting vasopressin analogue, also produces a similar effect.[2,7] This phenomenon has been proposed to be attributed to the excessive secretion of vasoconstrictors or toxic metabolites during hypovolemia.[8,9]

Portal hypertension with hyperdynamic circulation is manifested by vasodilatation, increased systemic and regional blood flows, and enhanced cardiac performance, including tachycardia and elevated cardiac output.[10,11] Nitric oxide (NO) and prostacyclin play a role in the development and maintenance of hyperdynamic circulation and vascular hyporesponsiveness to vasoconstrictors in portal hypertensive states,[12-19] as well as the vascular hyporeactivity in hemorrhagic shock.[20,21] Inhibition of NO and prostacyclin synthesis overcame the

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Correspondence to: Fa-Yauh Lee, MD, F.A.C.G., Division of Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, 201, Sec 2, Shih-Pai Road, Taipei 112, Taiwan.
Tel: +886-2-2875-7308; Fax: +886-2-2873-9318; E-mail: fylee@vghtpe.gov.tw

splanchnic hyposensitivity to glypressin in portal hypertensive rats with bleeding.[22,23] Recently, the over-expression of cyclooxygenase-1 in superior mesenteric artery participating in the aforementioned splanchnic hyporeactivity has been demonstrated.[24] However, the molecular basis of NO synthase (NOS) activation over systemic and splanchnic vasculatures remains to be surveyed.

This study investigated the impact of acute hemorrhage on the hemodynamic effects of glypressin and mRNA expression of constitutive NOS (cNOS) and inducible NOS (iNOS) in systemic and splanchnic vasculatures.

METHODS

Animal model

Male Sprague-Dawley rats weighing 300-350 gm were used for this study. The rats were caged at 24 °C, with a 12-h light-dark cycle and free access to food and water until the time of experiments. Survival surgery and hemodynamic study were performed with the rats under anesthesia with ketamine hydrochloride (100 mg/kg body weight intramuscularly). Portal hypertension was induced by partial portal vein ligation.[25] In brief, the portal vein was isolated and a 3-0 silk ligature was tied around both the portal vein and an adjacent 20-gauge blunt-tipped needle. The needle was then removed and the vein allowed to reexpand. A second loose ligature was left around the portal vein with 2 endings of the ligature placed on each side in the abdominal cavity. The abdomen was then closed and the animal allowed to recover. The experiments reported here were conducted according to the American Physiological Society guiding principles for the care and use of laboratory animals.

Experimental design (Fig. 1)

On the 14 th day after portal vein ligation (PVL), rats were randomly divided into without- or with-bleeding groups. To avoid confounding factors, the rats were randomly used in 3 series of experiments to investigate (1) the hemodynamic effects of glypressin (0.07 mg/kg intravenously for 1 minute; without-bleeding, n = 6; with-bleeding, n = 6), and (2) cNOS/iNOS mRNA expression over abdominal aorta and superior mesenteric artery (without-bleeding, n = 14; with-bleeding, n = 14). Blood was withdrawn for 15 min at a constant rate of 0.3 mL/min from those rats subjected to hemorrhage.[7,26] After a 20-min stablization period, 50% of the withdrawn blood was re-infused for 7.5 min at the same rate as that in hemorrhage. The infusion and withdrawal of blood were performed using the infusion/withdrawal pump (model SP 210 iw, World Precision Instruments, Sarasota, FL, USA) *via* a PE-50 catheter connected to the right carotid artery. In the without-bleeding group, no blood was withdrawn or re-infused in these 2 periods. Forty-five minutes later, the second hemodynamic measurement was performed, followed by glypressin (0.07 mg/kg, Ferring Company, Kiel, Germany) infusion for 1 minute with an infusion pump *via* a PE-50 catheter connected to the right jugular vein. Ten minutes after glypressin administration, the third hemodynamic study was done. The same hemorrhage-transfused steps were performed for the second and third series of rats except glypressin infusion and hemodynamic measurements. At the end of hemorrhage-transfused procedures, abdominal aorta and superior mesenteric artery were dissected and removed for RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) to semi-quantitate the cNOS and

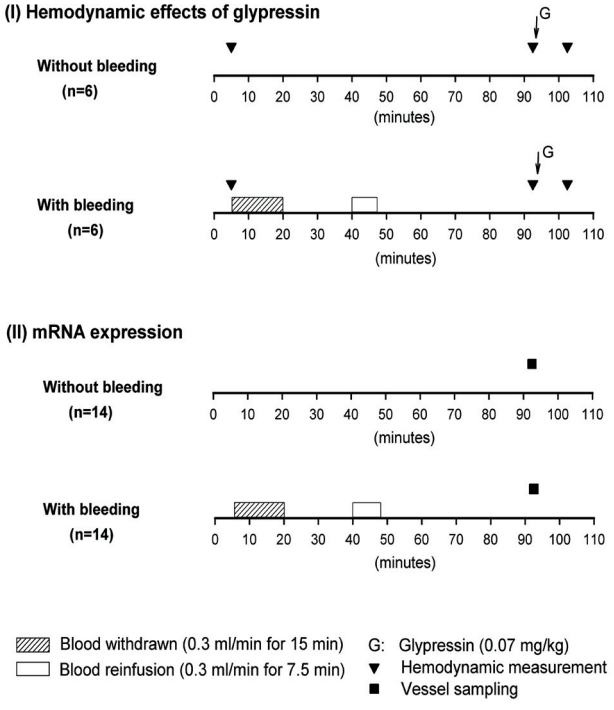

Fig. 1. Experimental design.

iNOS expression in the second series.

Measurement of systemic and portal hemodynamics

The right femoral artery of PVL rats was cannulated with a PE-50 catheter that was connected to a Spectramed DTX transducer (Spectramed Inc., Oxnard, CA, USA). Continuous recordings of mean arterial pressure, heart rate and portal pressure were performed on a multi-channel recorder (model RS 3400, Gould Inc., Cupertino, CA, USA). The external zero reference was placed at the level of the mid-portion of the rat. Heart rate was determined from the recording. The abdomen was then opened with a mid-line incision, and a mesenteric vein was cannulated with a PE-50 catheter connected to a Spectramed DTX transducer. The abdominal cavity was closed and the portal pressure was recorded on a Gould model RS 3400 recorder.[7,27]

Ribonucleic acid isolation, RT and PCR

Total RNA was extracted from abdominal aorta and superior mesenteric artery, respectively with a RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.[28,29] A one-step RT-PCR kit (Qiagen GmbH, Hilden, Germany) was used with the following components: 10 μL of RT-PCR buffer containing Tris-HCl, KCl, $(NH_4)_2SO_4$ and 2.5 mM $MgCl_2$; 2 μL of deoxynucleotide mixture containing 400 μM of dATP, dCTP, dGTP, and dTTP, respectively; 2 μL of enzyme mixture containing Omniscript™ reverse transcriptase, Sensiscript™ reverse transcriptase, and HotStar Taq™ DNA polymerase; 1 μL of RNase inhibitor (40 U); 3 μL of each random primers (10 ρmol/μL), and 1 μg of substrate RNA. Rnase-free water was added in each reaction to the final volume of 50 μL. The sequences of primers for cNOS were 5'-TACGGAGCAGCAAATCCAC-3' (sense) and 5'-CAGGCTGCAGTCCTTTGATC-3' (antisense), respectively. Those for iNOS were 5'-CCCTTCCGAAGTTTCTGGCAGCAG-3' (sense) and 5'-GGGCTCCTCCAAGGTGTTGCCC-3' (antisense), respectively. A constitutively expressed gene, β-actin, was analyzed as control, and the primers for β-actin were 5'-TTGTAACCAACTGGGACGATATGG-3' (sense) and 5'-GATCTTGATCTTCATGGTGCTAGG-3' (antisense), respectively. A negative control of the reaction was included in each set of experiments. The tubes were placed in the thermocycler (Biometra® T Gradient thermocycler, Biometra GmbH, Göttingen, Germany) at 50 °C for 30 minutes for reverse transcription, 95 °C for 15 minutes for initial denaturation, followed by 35 cycles of the following sequential steps: 30 seconds at 94 °C (denaturation), 45 seconds at 57.2 °C for cNOS, 62 °C for iNOS, 59.6 °C for β-actin (annealing), respectively and 45 seconds at 72 °C (extension). The final extension was performed at 72 °C for 10 minutes. The primers for cNOS, iNOS and β-actin were designed to allow amplification of 819, 473, and 764 base-pair fragments, respectively. Ten μL of the PCR-amplified mixture was subjected to electrophoresis on a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining. Location of the predicted PCR products (base pairs) was confirmed by using a 100-base pair DNA ladder (GIBCO BRL, Gaithersburg, MD, USA) as standard size maker. The gel was then photographed and the signal intensity of β-actin, iNOS and cNOS products respectively was quantitated by a digitalized software (Kodak Digital Science™ ID Image Analysis Software, Eastman Kodak Co., Rochester, NY, USA). The index of cNOS and iNOS signal was standardized against that of β-actin signal from the same RNA sample and expressed as cNOS/β-actin and iNOS/β-actin ratio for comparison.

Statistical Analysis

Results are expressed as mean ± SE. Statistical analyses were performed using two-sample Student's *t* test. Results were considered statistically significant when a p value < 0.05 was found.

RESULTS

Hemodynamic effects of glypressin

There was no significant difference in the mean arterial pressure (99.3 ± 8.1 *vs*. 105.7 ± 4.1 mmHg, p = 0.503), portal pressure (11.6 ± 0.5 *vs*. 12.0 ± 0.9 mmHg, p = 0.724), and heart rate (303 ± 32 *vs*. 312 ± 21 beats/min, p = 0.821) between the without-bleeding and with-bleeding groups at baseline.

After the procedure of blood withdrawal/re-infusion and before the administration of glypressin, there

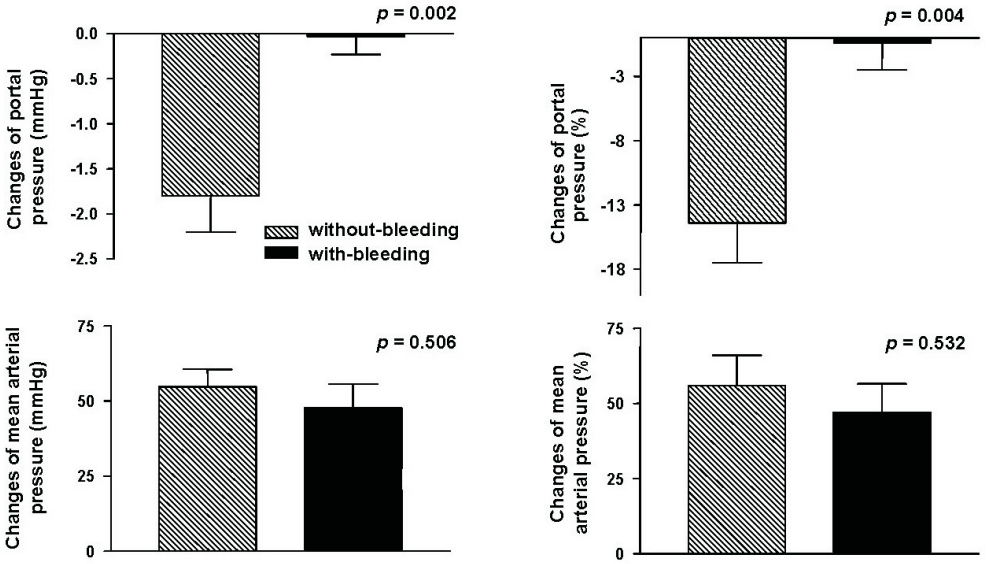

Fig. 2. Effects of glypressin infusion on mean arterial pressure and portal pressure in the without- and with-bleeding PVL rats.

was no difference in the mean arterial pressure (without-bleeding *vs*. with-bleeding group: 104.6 ± 8.1 *vs*. 105.8 ± 6.0 mmHg, $p = 0.910$) and heart rate (351 ± 35 *vs*. 322 ± 22 beats/min, $p = 0.502$) between without- and with-bleeding PVL rats. However, the portal pressure was significantly lower in the with-bleeding group (12.6 ± 0.4 *vs*. 11.0 ± 0.5 mmHg, $p = 0.031$).

The portal-hypotensive effect of glypressin showed blunted in the with-bleeding group when compared with the without-bleeding group (changes of portal pressure: without-bleeding *vs*. with-bleeding group: -1.8 ± 0.4 *vs*. -0.03 ± 0.2 mmHg, $p = 0.002$; percentage/percent changes of portal pressure: -14.4 ± 3.1% *vs*. -0.4 ± 2.1%, $p = 0.004$, Fig. 2). The increase of mean arterial pressure (54.7 ± 5.9 *vs*. 47.8 ± 7.9 mmHg, $p = 0.506$; 56.0 ± 10.0% *vs*. 47.1 ± 9.5%, $p = 0.532$, Fig. 2) and change of heart rate (-3 ± 15 *vs*. -7 ± 15 beats/min, $p = 0.881$; 0.5 ± 5.1% *vs*. -2.5 ± 5.2%, $p = 0.699$) after glypressin infusion were similar between the without-bleeding and with-bleeding groups.

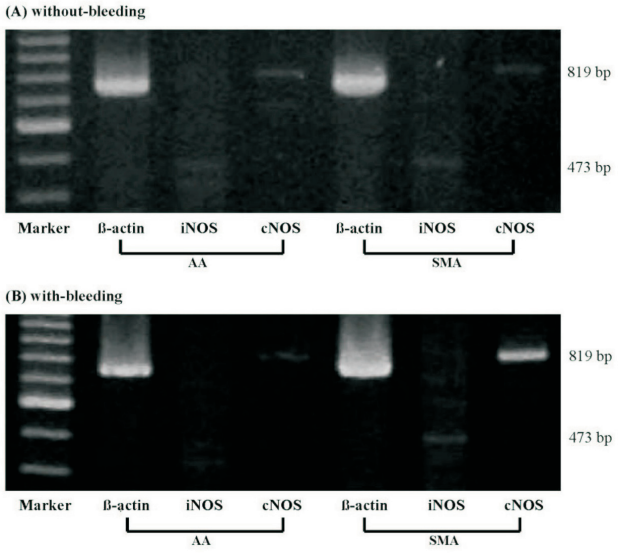

Fig. 3. Agarose gel electrophoresis of iNOS and cNOS cDNA in superior mesenteric artery (SMA) and abdominal aorta (AA) of without- and with-bleeding PVL rats (Marker: 100-base pair DNA ladder; bp: base pairs; iNOS: 473 bp; cNOS: 819 bp).

cNOS/iNOS expression

cNOS mRNA expression was significantly higher in the superior mesenteric artery of the with-bleeding group (cNOS/β-actin of without-bleeding *vs*. with-bleeding group: 0.16 ± .03 *vs*. 0.28 ± .04, $p = 0.021$), whereas no significant changes were found in cNOS (cNOS/β-actin: 0.15 ± 0.03 *vs*. 0.17 ± 0.03, $p = 0.703$) and iNOS expression (iNOS/β-actin: 0.05 ± 0.02 *vs*. 0.04 ± 0.01, $p = 0.174$) in abdominal aorta and iNOS expression (iNOS/β-actin: 0.03 ± 0.00 *vs*. 0.06 ± 0.02, $p = 0.143$) in the superior mesenteric artery (Figs. 3 and 4).

DISCUSSION

Similar to our previous reports,[7,23,30] the current study

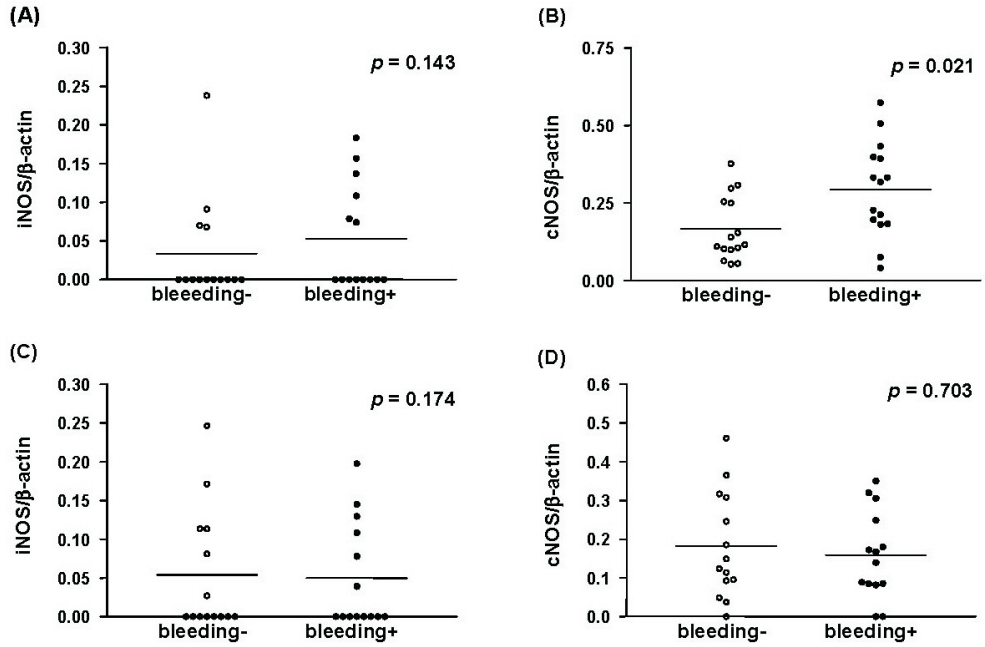

Fig. 4. Agarose gel electrophoresis of iNOS and cNOS cDNA in superior mesenteric artery (A and B) and abdominal aorta (C and D) of without- (bleeding-) and with-bleeding (bleeding+) PVL rats.

demonstrates that during acute bleeding, glypressin induced a significant elevation in the mean arterial pressure of PVL rats without remarkable influence on portal pressure. In PVL rats without bleeding, however, glypressin administration achieved similar effect on the mean arterial pressure but effectively reduced portal pressure. These results suggest the existence of splanchnic hyposensitivity to glypressin in bleeding PVL rats, similar to the hyporesponsiveness phenomenon with vasopressin infusion.[3,4]

NO has been demonstrated to mediate an attenuated response to various vasoconstrictors and contribute to the vascular hyporeactivity to arginine vasopressin in portal hypertensive states.[14,31] In addition, NO participates in the splanchnic hyposensitivity to glypressin in hemorrhage-transfused PVL rats, and NO synthesis inhibition by N^G-nitro-L-arginine methyl ester (L-NAME) significantly improved the aforementioned hyporeactivity.[7,22,30] NO is a potent vasodilator produced locally in the vessel wall from L-arginine[32] synthesized by various key enzymes with 2 distinct isoforms: cNOS and iNOS. In many cell types, cNOS synthesizes NO to maintain basal vascular tone and physiological activities, whereas iNOS is transiently induced by serum, growth factors, cytokines, tumor necrosis factor, endotoxin, and other factors.[33] In the present study, the cNOS expression of the superior mesenteric artery after hemorrhage was significantly higher than that of the non-bleeding group, suggesting the predominant role of cNOS in maintaining splanchnic blood flow. During status with hemorrhagic shock, enhanced release of NO by cNOS has been suggested, since it was reversed by L-NAME, a relatively non-selective NOS inhibitor, but was not prevented by dexamethasone, an inhibitor of iNOS.[20] Similar observations showing evidence that cNOS suppression reversed the splanchnic hyposensitivity in PVL rats during acute hemorrhage have also been demonstrated in our previous study.[30] As mesenteric vascular resistance increases greatly as hemorrhage progresses,[34,35] cNOS over-expression suggests its role in maintaining splanchnic blood flow, thus mediating hyporesponsiveness to vasoconstrictors in portal hypertensive model with acute bleeding. This is demonstrated by our current results that mRNA expression of cNOS rather than iNOS in superior mesenteric artery was enhanced during acute hemorrhage. In addition, cNOS and iNOs expressions did not change in aorta.

Prostacyclin (PGI_2) and NO are 2 major endogenous vasodilators that participate in the splanchnic hyperemia,[15-17] and splanchnic vascular hyporeactivity to vasoconstrictor associated with portal hypertension.[13,14,18,19] Besides NO, PGI_2 has also been shown to mediate the

splanchnic hyposensitivity to glypressin in hemorrhage-transfused PVL rats.[7,22,30] Among the isoenzymes responsible for PGI_2 synthesis under such a setting, cyclooxygenase-1 over-expression in the superior mesenteric artery has been found.[24] In addition, hemorrhage is associated with the endogenous release of brakykinin (a cNOS activator),[36,37] and the influence of brakykinin on splanchnic hyposensitivity to glypressin during acute hemorrhage has been demonstrated.[30] Apart from constitutive NO synthase activation and prostacyclin, endothelium-derived hyperpolarizing factor has also been reported to be involved in the vasodilatory action of bradykinin.[38,39] Therefore, the splanchnic glypressin hyposensitivity seems to be mediated by multiple vasodilators.

In conclusion, splanchnic hyposensitivity to glypressin was found in portal hypertensive rats during acute hemorrhage. This condition may be related to increased synthesis of NO by cNOS activation in splanchcnic vascular beds, which has important implications for administering cNOS inhibitor to correct such a vascular hyporeactivity at an earlier stage.

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