Involvement of Constitutive Nitric Oxide Synthase in the Portal-systemic Collaterals of Portal Hypertensive Rats

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Background: Recent studies have shown that endothelial nitric oxide (NO) is involved in modulating the vascular response to vasoconstrictors in portal-systemic collaterals of portal hypertensive rats. This study investigated which isoform of NO synthase is involved in the collateral circulation of portal hypertensive rats.

Methods: The relaxation response to acetylcholine (10^{-8} M, 10^{-7} M and 10^{-6} M) in norepinephrine (NE)-preconstricted portal-systemic collaterals was investigated after incubation with vehicle (Krebs solution), a preferential inducible NO synthase inhibitor (aminoguanidine [AG]), or a non-selective NO synthase inhibitor (N^{ω}-nitro-L-arginine [NNA]), in rats with partial portal vein ligation. Mean arterial pressure was measured before the perfusion experiments.

Results: Bodyweight and mean arterial pressure before the perfusion studies were similar in the vehicle, AG and NNA groups. Preincubation with NNA, but not AG, produced a significant increase in baseline perfusion pressure compared with the vehicle group ($p < 0.05$). The increase in perfusion pressure in response to NE was enhanced in the presence of NNA ($p < 0.05$), but not AG. In addition, preincubation with NNA, but not AG, significantly suppressed acetylcholine-induced relaxation in the portal-systemic collaterals ($p < 0.05$).

Conclusion: These results suggest that constitutive, rather than inducible, NO synthase is involved in the vascular response to vasoconstrictors in the portal-systemic collaterals of portal hypertensive rats. [*J Chin Med Assoc* 2005; 68(6):245–249]

Key Words: aminoguanidine, N^{ω}-nitro-L-arginine, nitric oxide, portal hypertension, portal-systemic collaterals

Introduction

Portal hypertension and portal-systemic shunting are clinical features in patients with liver cirrhosis. The development of a portal-systemic collateral circulation may lead to lethal complications, such as hepatic encephalopathy, gastroesophageal varices, and variceal bleeding. The pathogenesis of collateralization is unknown, but may be related to the presence of portal hypertension and hyperdynamic circulation.[1]

Nitric oxide (NO) is a potent vascular endothelium-derived vasodilator produced from L-arginine. It can be synthesized by various key enzymes, with 2 distinct isoforms: constitutive NO synthase (cNOS) and inducible NO synthase (iNOS).[2] In many cell types, cNOS synthesizes NO to maintain basal vascular tone and physiologic homeostasis, whereas iNOS is transiently induced by serum, growth factors, cytokines, tumor necrosis factor, endotoxins, and other factors.[3] Chronic administration of N^{ω}-nitro-L-arginine (NNA), a non-selective NOS inhibitor, ameliorates portal-systemic shunting in portal hypertensive rats, which suggests that NO plays a role in collateralization of the portal system.[1] Recently, an *in situ* perfusion model of

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the collateral circulation was developed successfully in rats with partial portal vein ligation (PVL).[4] Using this model, some authors demonstrated that NO is involved in modulating the vascular response to vasoconstrictors in the portal-systemic collaterals of rats with PVL.[5,6] However, the contribution of NOS isoforms in the collateral circulation remains unknown.

In the present study, NNA and aminoguanidine (AG; a preferential iNOS inhibitor)[7,8] were used to clarify which isoform of NOS is involved in the collateral circulation of portal hypertensive rats.

Methods

Study animals

Adult male Sprague-Dawley rats, weighing 300–350 g at the time of surgery, were used for study. The rats were housed in a plastic cage in a 12-hour light-dark cycle, and were allowed free access to food and water until the time of the experiments. All rats were fasted for 12 hours before operation. In all experiments, the investigators adhered to American Physiological Society Guiding Principles for the Care and Use of Laboratory Animals. Survival surgery was performed under ether anesthesia, followed by intramuscular ketamine hydrochloride (100 mg/kg bodyweight). Portal hypertension was induced by partial PVL, as previously reported.[9] In brief, the portal vein was isolated and a 3-0 silk ligature was tied around the portal vein and an adjacent 20-gauge blunt-tipped needle. The needle was then removed, allowing the vein to re-expand. A second loose ligature was left around the portal vein, with two endings of the ligature placed on each side in the abdominal cavity. The abdomen was then closed and the animal was allowed to recover. Perfusion studies were performed in overnight-fasted rats 10–13 days after the operation, when extensive collateralization of the portal system was fully established.[10] Bodyweight and mean arterial pressure of the rats were measured on the day of perfusion studies.

In situ perfusion of portal-systemic collaterals

In situ perfusion studies were performed as previously described.[4,5] Both jugular veins were cannulated with 16-gauge Teflon cannulas to ensure an adequate outflow, without resistance even at the highest flow rates. Heparin (200 U/100 g bodyweight) was injected through one of the cannulas. The abdomen was then opened and an 18-gauge Teflon cannula inserted in the distal mesenteric vein and fixed with cyanoacrylate glue. To prevent liver perfusion, a second loose ligature was tied around the portal vein. The animal was transferred into a warm chamber (37 ± 0.5°C). The temperature around the perfusion area was continuously monitored, with a thermometer placed inside the mesentery, and was maintained at approximately 37 ± 0.5°C with a thermostatic pad and temperature-controlled infrared lamp. Open-circuit perfusion was then started with Krebs solution (composition in mmol/L: NaCl 118; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; dextrose 11.0; pH 7.4; 37 ± 0.5°C) via the mesenteric cannula by a roller pump (model 505S, Wastson-Marlow Ltd, Falmouth, Cornwall, England). The perfusate was equilibrated with carbogen gas (95% O_2, 5% CO_2) by a silastic membrane lung.[11] Both jugular vein cannulas were simultaneously opened to allow a complete washout of the blood. Pneumothorax was created by opening slits through the diaphragm to increase resistance in the pulmonary artery and prevent perfusate from entering the left heart chambers.

The portal-systemic collaterals were then perfused with oxygenated (95% O_2, 5% CO_2) Krebs solution containing 3% w/v albumin (factor V bovine serum albumin; Sigma Chemical Co, St. Louis, MO, USA). The effluent of the perfused tissue was collected in a reservoir and was not recirculated. To monitor and continuously record pressure in the portal-systemic collaterals, a Spectramed DTX transducer attached to the Gould model RS 3400 recorder (Gould Inc, Cupertino, CA, USA) was connected to a side arm placed just proximal to the perfusion cannula, with the 0 placed at the level of the right atrium. As temperature and pressure of the system stabilized within 20 minutes, all experiments were performed 30 minutes after starting perfusion at a constant rate of 20 mL/min. Since the perfusion flow rate was kept constant throughout the experiment, changes in perfusion pressure reflected changes in collateral vascular resistance.

Experimental design

The relaxation response of acetylcholine (ACh) in norepinephrine (NE)-preconstricted portal-systemic collaterals in the presence or absence of AG or NNA was investigated. Three groups of rats were used: vehicle (Krebs solution) plus NE and ACh ($n = 7$); AG (10^{-4} M) plus NE and ACh ($n = 6$); and NNA (10^{-4} M) plus NE and ACh ($n = 7$). In the experiment, portal-systemic collaterals were preconstricted with 0.3 μM NE, a concentration causing a half maximal effect,[4] to evaluate the relaxation response to ACh. To retard the oxidation of NE, ascorbate 0.2 μM was used. ACh (10^{-8} M, 10^{-7} M and 10^{-6} M) was introduced into the perfusate 5 minutes after challenge with NE, and each

concentration was allowed to stabilize for 3 minutes before the next higher concentration was added. NOS inhibitors or vehicle were introduced into the perfusate 15 minutes before the NE challenge and were present throughout subsequent stimulations. Only 1 concentration-response curve was performed for each preparation.

Drugs

The reagents for preparing Krebs solution, NE, ACh, AG, and NNA were purchased from Sigma Chemical Co. All drugs were prepared freshly for each experimental day.

Statistical analysis

Results are expressed as mean ± standard error. Changes in perfusion pressure from baseline were calculated for each concentration of each preparation. Statistical analyses were performed using the paired or unpaired Student's *t* test, and 1-way analysis of variance with Tukey's test when appropriate. A *p* value of less than 0.05 was considered statistically significant.

Results

Table 1 shows the baseline characteristics of rats in the different groups studied. No significant, between-group differences were observed in bodyweight, number of days of PVL, and mean arterial pressure. Preincubation with NNA, but not AG, significantly increased baseline perfusion pressure relative to the vehicle group (NNA vs AG and vehicle: 20.3 ± 0.8 vs 12.6 ± 0.8 and 13.6 ± 1.6 mmHg, respectively; $p < 0.05$). In the vehicle group, NE preconstriction significantly elevated perfusion pressure from baseline (change from baseline perfusion pressure: 4.8 ± 0.6 mmHg; $p < 0.05$). The above-baseline increase in perfusion pressure in response to NE was significantly enhanced in the presence of NNA (7.4 ± 1.0 mmHg; $p < 0.05$), but not AG (4.5 ± 0.6 mmHg) (Figure 1).

Table 2 shows ACh-induced percent changes in perfusion pressure (from NE-induced preconstriction) in portal-systemic collaterals. ACh induced progressive, concentration-dependent relaxation of initial NE-induced constriction in the vehicle group.

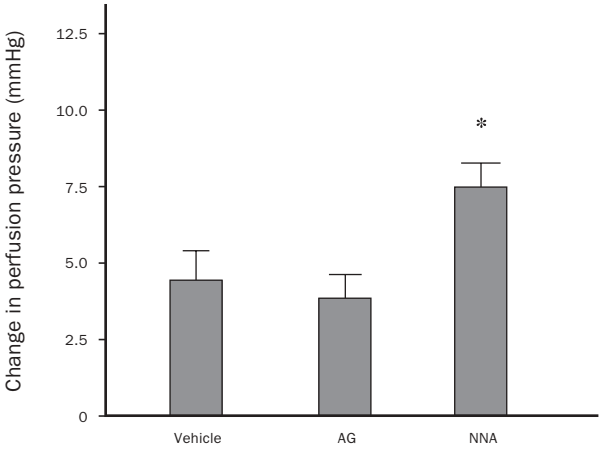

Figure 1. Change in perfusion pressure in portal-systemic collaterals after challenge with norepinephrine, with or without aminoguanidine (AG) or N^{ω}-nitro-L-arginine (NNA). *$p < 0.05$ vs AG and vehicle.

Table 1. Baseline characteristics of rats in the different study groups

Characteristic	Vehicle (n = 7)	AG (n = 6)	NNA (n = 7)
Bodyweight (g)	351.6 ± 15.2	347.8 ± 5.7	319.4 ± 11.3
Days of PVL	11.7 ± 0.8	11.4 ± 1.2	10.1 ± 0.9
MAP (mmHg)	86.8 ± 4.4	82.3 ± 7.5	86.8 ± 4.3
BPP (mmHg)	13.6 ± 1.6	12.6 ± 0.8	20.3 ± 0.8*

**p < 0.05 vs AG and vehicle. AG = aminoguanidine; BPP = baseline perfusion pressure; MAP = mean arterial pressure; NNA = N^{ω}-nitro-L-arginine; PVL = partial portal vein ligation.*

Table 2. ACh-induced percent changes in perfusion pressure (from NE-induced preconstriction) in portal-systemic collaterals in the different study groups

	Vehicle (n = 7)	AG (n = 6)	NNA (n = 7)
10^{-8} M ACh	–14.3 ± 2.6	–11.6 ± 0.7	–8.0 ± 2.0
10^{-7} M ACh	–37.4 ± 3.5	–32.4 ± 5.6	–15.0 ± 4.3*
10^{-6} M ACh	–51.4 ± 5.4	–44.9 ± 7.2	–14.2 ± 5.7*

**p < 0.05 vs AG and vehicle. Values shown are mean ± standard error percent changes in perfusion pressure relative to NE-induced preconstriction. ACh = acetylcholine; AG = aminoguanidine; NNA = N^{ω}-nitro-L-arginine.*

Preincubation with NNA, but not AG, significantly suppressed ($p < 0.05$) ACh-induced relaxation in the portal-systemic collaterals.

Discussion

The *in situ* model of portal-systemic collateral perfusion used in this study is a stable and highly reproducible model that can be used to investigate the response of portal-systemic collaterals to various pharmaceutical agents in portal hypertensive status.[4–6] In addition, for pharmacologic study, it is a highly sensitive and reproducible technique for evaluating the collateral vasculature. This collateral-perfusion technique demonstrated that the flow-pressure slope (i.e. vascular resistance in the portal-systemic collateral vasculature) decreased significantly and progressively from days 2–7 after induction of portal hypertension in the rats;[4] this is compatible with observations using the microsphere method.[10] The *in situ* perfusion model evaluates resistance to flow across all portal-systemic collateral vessels, whereas the radioactive microsphere technique cannot evaluate vessels with a diameter greater than that of the microspheres (~15 ± 0.1 μm). In addition, the non-recirculating perfusion system excludes the influence of vasoactive substances released from anoxic organs during experiments.[5]

According to a previous study by Mosca et al,[4] concentration-dependent relaxation to ACh concentrations of 10^{-8}–10^{-6} M was exhibited in the perfused collaterals of portal hypertensive rats. Maximal ACh-induced vasodilation reduced NE-induced preconstriction by about 50%. As such vasodilation is mediated by NO, and as NO is an endothelium-derived vasodilatory substance, the study also tested the role of the endothelium in the vasodilatory effect of ACh. Results showed that removal of the portal-systemic collateral vascular endothelium, with sodium cholate 1%, blunted the vasodilatory effect of ACh.[4] In the present study, which surveyed the relative contribution of different NOS subtypes, vehicle-treated rats had a concentration-dependent relaxation to ACh. The non-selective NOS inhibitor NNA significantly diminished ($p < 0.05$) the vasodilatory response to ACh, even at an ACh concentration of 10^{-6} M (–14.2% with NNA, vs –51.4% and –44.9% with vehicle and AG, respectively). However, a similar effect was not observed with AG, a preferential iNOS inhibitor. These results suggest that the enhanced NO activity in the collateral vascular bed of portal hypertensive rats is due to cNOS rather than iNOS.

In the present study, NE preconstriction significantly increased perfusion pressure in portal-systemic collaterals in the vehicle group. Preincubation with NNA not only increased baseline perfusion pressure, but also potentiated NE-induced vasoconstriction. This finding is consistent with the previous report by Mosca et al.[4] Preincubation with NNA also enhanced the vasoconstrictive effects of vasopressin and endothelin on the portal-systemic collaterals in portal hypertensive rats, as demonstrated in previous studies.[5,6] However, the preferential iNOS inhibitor AG, unlike NNA, had no major effects on baseline perfusion pressure or NE-induced vasoconstriction in the portal-systemic collaterals.

Considerable debate exists about which NOS isoforms are responsible for the development of hyperdynamic circulation in portal hypertension. With measurements of cNOS and iNOS activity, Fernándes et al[12] demonstrated that iNOS is not required for development of the hyperkinetic systems and splanchnic circulation in PVL or cirrhotic rats. In addition, Soubrane et al[13] observed no iNOS gene expression in the aorta of cirrhotic or PVL rats. However, cloning studies in experimental portal hypertension have demonstrated iNOS mRNA expression in the arteries of cirrhotic rats,[14,15] and in the endothelia of esophageal submucosal veins in PVL rats.[16] Recently, cNOS, but not iNOS, expression was found in the thymus, aorta, heart, lung, esophagus, liver, spleen, kidney, pancreas, and small and large intestine.[17] In addition, using cNOS or iNOS gene-knockout mice, Theodorakis et al[18] reported that cNOS, rather than iNOS, is important in the pathogenesis of portal hypertension. Our study also demonstrated a lack of iNOS activity in the collateral circulation, and all these observations suggest that it is primarily cNOS that contributes to increased NO activity in the collateral vascular bed in portal hypertension.

Regarding the effect of NO on portal-systemic collateral formation, it has been found that NO inhibition ameliorates portal-systemic shunting in portal hypertensive rats.[1,19] Recently, the role of NO as an autocrine regulator of the microvascular events necessary for angiogenesis has also been reported,[20,21] which supports the notion that cautious NO inhibition may contribute to improved control over varices formation in patients with liver cirrhosis. The relative contribution of NOS isoforms also requires further delineation.

In conclusion, preincubation with a non-selective NOS inhibitor, NNA, but not the preferential iNOS inhibitor, AG, enhanced NE-induced vasoconstriction and suppressed ACh-induced vasodilatation in portal-

systemic collaterals in portal hypertensive rats. These findings suggest that the main source of NO production in the collateral circulation depends on cNOS activity.

Acknowledgments

The authors gratefully acknowledge Chun-Ching Tai, Shwu-Ling Wu, and I-Nien Lai for their excellent technical assistance. This work was supported by grants from the National Science Council (NSC93-2314-B-075-060) and Taipei Veterans General Hospital (VGH93-212), Taiwan, R.O.C.

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