Abstract—During the course of our studies into the control of fluid extravasation from the splenic vasculature, we found that intrasplenic inhibition of NO biosynthesis caused an increase in systemic blood pressure. The present experiments were designed to investigate the mechanisms underlying this novel observation. There was an increase in mean arterial pressure when the nonspecific NO inhibitor $N^\epsilon$-monomethyl-L-arginine (L-NMMA) was infused via the splenic artery but not when the same dose was administered systemically. Conversely, blood pressure decreased after intrasplenic but not systemic administration of the NO donor S-nitroso-N-acetyl-D,L-penicillamine. There was no pressor response to intrasplenic administration of either the inducible or neuronal NO synthase inhibitors $N$-[3-(aminomethyl)-benzyl] aceramidine and $L$-Nm(1-imino-3-butenyl)-ornithine. The pressor response to L-NMMA was abolished by denervation of either the spleen or the kidney and by pretreatment with the ACE inhibitor enalapril. We propose that the spleen influences systemic blood pressure through a reflex pathway comprising splenic afferent nerves and renal sympathetic control of renin release. (Hypertension. 2001;38:348-352.)

Key Words: spleen ■ kidney ■ renin ■ nitric oxide ■ blood pressure

We have shown that protein-rich fluid is filtered out from the splenic circulation into the systemic lymphatic system and that this mechanism is important in controlling intravascular volume under both physiological and pathological conditions, such as endotoxemia. During the course of our studies into the effects of NO on splenic function, we found that intrasplenic infusion of very small doses of the NO synthase (NOS) inhibitor $N^\epsilon$-monomethyl-L-arginine (L-NMMA) increased systemic blood pressure. The experiments presented in this article were designed to investigate the mechanisms underlying this observation.

Methods

The experiments described here were examined by the local Animal Welfare Committee and found to be in compliance with the guidelines issued by the Canada Council on Animal Care. At the completion of the studies, all animals were killed with an anesthetic overdose (0.2 mL IV Euthanyl, MTC Pharmaceuticals).

Animals and Housing

Male Long Evans rats (450 to 600 g) were obtained from Charles River Canada (St Foy, Quebec). They were held in the University Animal Facility for ≥1 week before surgical or experimental procedures. The animal room was temperature and humidity controlled, with a 12-hour/12-hour light/dark cycle. The rats were maintained on a 0.3%-sodium diet (PMI Feeds) and water ad libitum.

Surgery

Under sodium pentobarbital anesthesia (62 mg/kg IP), the rats were implanted with femoral venous (Silastic; 0.51-mm ID, 0.94-mm OD) and arterial (PE 50; 0.58-mm ID, 0.97-mm OD) cannulae. The venous cannula was used for infusing saline and drugs; the arterial cannula was used for monitoring blood pressure. Through a midline laparotomy, a Silastic cannula (0.31-mm ID, 0.64-mm OD) was placed in the gastric artery so that its tip lay just above the junction with the splenic artery. The vascular arcade serving the spleen was isolated by ligating all vessels leading to or from other vascular beds. At the end of each experiment, dye was infused into the gastric artery to confirm that it perfused only the spleen, ie, that all blood passing through the flow probe did indeed exclusively supply the splenic circulation. Flow probes (size 1RB, Transonic Systems Inc) were placed around the splenic artery and vein. These flow probes are factory calibrated. The zero–blood-flow reading for each flow sensor was confirmed before implantation by placing the probe in a nonturbulent saline bath. Validation of the accuracy of blood flow measurements by these probes has previously been reported.

Splenic and Renal Denervation

With the aid of a surgical microscope, the splenic nerve bundle was located on the splenic artery and vein beneath the splenic lymph node. The nerve was isolated, and a 1.0-mm length was resected. The sham-control animals were subjected to the same procedure, except the nerve was not interrupted. We have confirmed in other experiments that even 7 days after denervation, this procedure reduces splenic tissue catecholamine levels from 222 ± 34 ng/mL (n = 10) to 32 ± 8 ng/mL (n = 10) as measured with a [$^3$H]radioenzymatic assay (Amersham Pharmacia Biotech; S. Kaufman, unpublished observation, 2000). The renal nerves were similarly isolated and sectioned. The blood vessels were then carefully painted with 5% phenol solution in alcohol to destroy any remaining nerves. We have previously confirmed that in our hands this procedure results in successful renal denervation. The splenic and renal denervations were done during the surgical preparation of the animals, ie, ~90 minutes before baseline readings of blood pressure were taken.

Drugs

The nonspecific NOS inhibitor L-NMMA (Calbiochem), the inducible NOS inhibitor $N$-[3-(aminomethyl)-benzyl] aceramidine

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Saline was infused through the femoral vein cannula at a rate of 3 mL/h. A supplementary dose of thiobutabarbital (60 mg/kg body weight SC) was administered to maintain a plane of anesthesia. Blood pressure was recorded online (Windaq, DATAQ Instruments Inc) through the femoral arterial cannula. After a 45-minute stabilization period, blood pressure and blood flows were recorded for 30 minutes. The last 5 minutes of this period was accepted for measurement of baseline values. Preliminary experiments established that L-NMMA in doses of 0.2 and 2.7 mmol/L · 10 μL⁻¹ · min⁻¹ significantly increased mean arterial pressure (MAP) when infused into the splenic artery. The lower dose was therefore chosen for subsequent experiments. The following protocols were followed.

In protocol A, L-NMMA (0.2 mmol/L · 10 μL⁻¹ · min⁻¹) or saline (control) was infused for 30 minutes. It was then infused with l-arginine (2.9 mmol/L · 10 μL⁻¹ · min⁻¹) or saline for an additional 45 minutes. Blood pressure was recorded online continuously. In some experiments, splenic arterial and venous blood flow was also recorded. In the experiments designed to investigate the effect of blocking the renin-angiotensin system, the rats were pretreated with enalapril. Preliminary experiments showed that the rats drank the enalapril. Preliminary experiments showed that the rats drank 91 ± 2 mL/d. At a concentration of 250 mg/L, the daily dose of enalapril was about 20 mg · kg⁻¹ · d⁻¹. This dosing regime has been previously validated in rats.⁶,⁷

In protocol B, in randomized order, each dose of SNAP (45.4, 136, and 454 μmol/L · 10 μL⁻¹ · min⁻¹) was infused for 15 minutes, followed by a 15-minute interval of vehicle infusion, the last 5 minutes of which was considered for measurement of baseline pressure for the next dose. In protocol C, 1400W (200 μmol/L · 10 μL⁻¹ · min⁻¹) or vinyl-LNIO (200 μmol/L · 10 μL⁻¹ · min⁻¹) was infused for 60 minutes.

### Study Groups

Preliminary experiments were performed with the high dose of L-NMMA (2.7 mmol · L⁻¹ · min⁻¹, n=4). Nine groups were subsequently run with the low dose of L-NMMA (0.2 mmol · L⁻¹ · min⁻¹) according to protocol A: (1) intrasplenic L-NMMA (n=8), (2) intrasplenic L-NMMA followed by intrasplenic l-arginine after 30 minutes (n=13), (3) femoral venous L-NMMA (n=6); (4) intrasplenic saline infusion (n=14), (5) splenic denervation plus intrasplenic L-NMMA (n=14), (6) splenic denervation plus intrasplenic saline (n=10), (7) renal denervation plus intrasplenic L-NMMA (n=8), (8) renal denervation plus intrasplenic saline (n=4), and (9) intrasplenic L-NMMA plus enalapril (n=4). In experiment B, there were 2 groups: (1) intrasplenic SNAP (n=7) and (2) femoral venous SNAP (n=8). In experiment C, there were 3 groups: (1) intrasplenic 1400W (n=8), (2) intrasplenic vinyl-LNIO (n=7), and (3) intrasplenic saline (n=6).

### Statistical Analysis

The basal MAP for the different groups was analyzed by ANOVA. The significance of changes across time was analyzed by 1-way repeated-measures ANOVA followed by the Dunnett test to identify the individual points of significance. If the data were not normally distributed, a repeated-measures ANOVA on ranks was used. The differences between L-NMMA–induced changes in vascular conductance were analyzed with Student’s t test for paired data. The differences between the intact and denervated animals were analyzed by 2-way ANOVA. The effect of enalapril on the L-NMMA–induced increase in blood pressure was analyzed by 2-way ANOVA. Significance was accepted at P<0.05.

### Results

There were no significant differences between the basal MAPs for the different groups: (1) intrasplenic L-NMMA, 94±4 mm Hg; (2) intrasplenic L-NMMA plus intrasplenic l-arginine, 88±2 mm Hg; (3) femoral venous L-NMMA, 98±6 mm Hg; (4) intrasplenic saline infusion, 95±2 mm Hg; (5) splenic denervation plus intrasplenic L-NMMA, 96±3 mm Hg; (6) splenic denervation plus intrasplenic saline, 94±2 mm Hg; (7) renal denervation plus intrasplenic L-NMMA, 98±2 mm Hg; (8) renal denervation plus intrasplenic saline, 87±8 mm Hg; and (9) intrasplenic L-NMMA plus enalapril, 88±6 mm Hg.

Preliminary experiments established that intrasplenic L-NMMA in doses of 0.2 mmol · L⁻¹ · 10 μL⁻¹ · min⁻¹ (n=8) or 2.7 mmol · L⁻¹ · 10 μL⁻¹ · min⁻¹ (n=4) significantly increased MAP (Figure 1). Although conductance fell in response to both doses of L-NMMA, the decrease was more pronounced at the higher dose, so that there was a substantial fall in splenic arterial blood flow (Table). The lower dose of L-NMMA was chosen for subsequent experiments. Given that splenic arterial blood flow was ~2 mL/min, it may be calculated that the concentration of L-NMMA entering the spleen was ~0.1 mol/L.

Intrasplenic infusion of L-NMMA caused a significant increase in MAP (Figures 1 and 2). In contrast, when it was infused through the femoral vein, L-NMMA (0.2 mmol · L⁻¹ · 10 μL⁻¹ · min⁻¹) had no effect on MAP (Figures 1 and 2B). SNAP caused a dose-dependent decrease in MAP when infused through the splenic artery but similarly no response when infused through the femoral vein (Figure 1).

The pressor response to L-NMMA was significant after 30 minutes of L-NMMA infusion and reached maximal levels by 60 minutes (Figure 2A). When, after 30 minutes, L-NMMA was coinfused through the splenic artery with l-arginine (2.9 mmol · L⁻¹ · 10 μL⁻¹ · min⁻¹), the increase in MAP was abolished (Figure 2A).
Neither the inducible NOS inhibitor 1400W nor the neuronal NOS inhibitor vinyl-LNIO caused any change in MAP when infused into the splenic artery (change in MAP from baseline at 60 minutes: 1400W, 2.4±0.6 mm Hg, n=8; L-VNIO, 1.4±2.0 mm Hg, n=7; and saline, 1.7±1.6 mm Hg, n=6).

Splenic denervation abolished the pressor response to L-NMMA (Figure 3A). Renal denervation also completely abolished the L-NMMA–induced increase in MAP (Figure 3B). Although splenic denervation alone caused a progressive hypotension, there was no such change in MAP after renal denervation. Although splenic blood flow did not change in the face of this fall in perfusion pressure (at 30 minutes, intact blood flow: 2.0±0.4 mL/min, n=8; splenic denervated blood flow: 2.0±0.3 mL/min, n=5), there was no significant difference in the calculated value of splenic conductance (intact: 0.022±0.003 mL · min⁻¹ · mm Hg⁻¹, n=8; splenic denervated animals: 0.026±0.003 mL · min⁻¹ · mm Hg⁻¹, n=5). Enalapril completely abolished the L-NMMA–induced increase in MAP (Figure 3C).

**Discussion**

Selective reduction in or augmentation of intrasplenic NO caused a respective increase or decrease in systemic blood pressure. The same doses of pharmacological agents were without effect when administered systemically. The increase in MAP in response to intrasplenic L-NMMA was abolished by interrupting either the splenic or the renal nerves. It was also inhibited by administration of the ACE inhibitor enalapril. On the basis of these results, we propose that the spleen participates in the regulation of blood pressure through a reflex pathway, whereby changes in splenic afferent nerve activity reflexly alter renal sympathetic control of renin...
release. The subsequent rise in circulating angiotensin II levels would act directly on the vasculature to raise total peripheral resistance. Angiotensin II could also alter central control of blood pressure by accessing the brain at the circumventricular organs.\(^8\)

There is both structural and functional evidence to support the existence of such a reflex pathway. Splenic afferent nerves have been demonstrated in the guinea pig and appear to be codistributed with substance P– and calcitonin gene–related peptide immunoreactive nerves.\(^9\) Histologically, they resemble unmyelinated C fibers.\(^10\) In dogs, stimulation of the splenic afferent nerves causes an increase in systemic blood pressure and increased renal and cardiopulmonary sympathetic nerve activity.\(^10\) This pathway may run, at least in part, through the brain, because stimulation of splenic afferent nerves also inhibits neurons in the rostral ventrolateral medulla.\(^11\) On the other hand, there is also evidence for spinal reflex pathways involving the splenic and renal nerves.\(^12\) Our finding that renal denervation and angiotensin II antagonism completely abolish the increase in blood pressure elicited by intrasplenic administration of L-NMMA suggests that the reflex is limited to the splanchic region, ie, that this stimulus does not elicit a generalized increase in sympathetic nervous system activity. In this regard, it is of interest that obstruction of the portal vein leads to an increase in efferent nervous discharge to the intestines, which does not originate from the liver and persists after transection of the spinal cord\(^13\); we suggest that this reflex could also arise from the spleen.

The stimulus for increased splenic afferent nerve activity does not seem to be directly related to intrasplenic pressure because occlusion of the celiac artery (decreased intrasplenic pressure) and manual splenic compression or occlusion of the splenic vein (increased intrasplenic pressure) induce excitation of the splenic afferent nerves.\(^10,14\) Flow rather than pressure may thus be a significant factor influencing splenic afferent nerve activity. In this regard, it is of significance that we observed evidence for this reflex after altering intrasplenic endothelium-derived NO. Under normal circumstances, endothelial NOS would be activated by an increase in blood flow and shear stress as that which would occur in response to a rise in splenic perfusion pressure.\(^15\) It might be anticipated that under these conditions there would be a homeostatic response to counter the change, ie, to lower blood pressure. It is conceivable that by mimicking such an increase in shear stress and NO biosynthesis with the NO donor SNAP, a reflex is initiated within the spleen to reduce systemic blood pressure. Conversely, a reduction in intrasplenic NO, mimicking reduced blood flow (the Table), would be expected to cause a reflex increase in blood pressure; this is what we observed. Our finding that neither the neuronal nor the inducible isofoms of NO synthase appear to be involved points to the importance of the endothelial NO system.

The question then arises as to how changes in intrasplenic NO alter splenic afferent nerve activity. There is evidence that NO, released from nonneuronal sources such as the intestinal vasculature during digestion, may inhibit slow excitatory postsynaptic potentials in the mesentery.\(^16\) Moreover, NOS inhibition or removal of NO within the celiac plexus prevents gastric distention–induced activation of the neurons and abolishes the gastroduodenal inhibitory reflex.\(^17\) It is suggested that NO inhibits neural activity by suppressing neurotransmitter release, but the mechanism by which this is achieved is not known.

We found that there was a progressive decline in MAP after splenic denervation (Figure 3A). Conceivably, this could be attributed to removal of splenic efferent (sympathetic) tone, intrasplenic vasodilatation, and a fall in total peripheral resistance. Although splenic blood flow did not decrease in parallel with the fall in perfusion pressure in the denervated animals (perhaps through autoregulation of blood flow), there was no significant difference in the calculated values of splenic vascular conductance between the intact and denervated animals. Even if with larger sample sizes the change in conductance had been significant (0.022 ± 0.003 mL · min\(^{-1}\) · mm Hg\(^{-1}\) versus 0.026 ± 0.003 mL · min\(^{-1}\) · mm Hg\(^{-1}\)), we would argue that this was not great enough to influence total peripheral resistance sufficiently to cause a fall in MAP. Thus, we would suggest that splenic afferent nerve activity provides tonic input to the maintenance of systemic blood pressure.

Although the finding that the splenorenal reflex controls blood pressure is novel, there has long been evidence for other spinal reflexes, such as the hepatorenal reflex. Increased intrahepatic pressure augments activity in the hepatic afferent nerves; this induces a rise in systemic blood pressure and in renal sympathetic efferent nerve activity.\(^18–20\) It has been suggested that the hepatorenal reflex is initiated by hepatic low-pressure baroreceptors.\(^21\) There is also ample evidence for a renorenal reflex through which increased intrarenal pressure, secondary to ureteral obstruction, activates renal mechanoreceptors, increases renal afferent nerve activity, reduces contralateral renal efferent nerve activity, and increases urine output from the contralateral kidney.\(^22\)

In conclusion, we have evidence for a splenorenal reflex through which increases or decreases in intrasplenic endothelial NO lower or raise MAP, respectively. We suggest that this may be mediated through changes in splenic afferent nerve activity, renal efferent nerve activity, renin release, and generation of angiotensin II. Physiologically, such alterations in intrasplenic NO could be elicited by changes in splenic blood flow and shear stress. Furthermore, our data suggest that the spleen exerts tonic control of systemic blood pressure. We propose that because the spleen drains into the hepatic portal vein, this splenorenal reflex could contribute to the homeostatic perturbations associated with such conditions as cirrhosis of the liver when splenic venous outflow pressure is raised.

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References
Splenoportal Reflex Regulation of Arterial Pressure
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