Sympathetic innervation of the splanchnic region mediates the beneficial hemodynamic effects of 8-OH-DPAT in hemorrhagic shock

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Tiniakov R, Pahan K, Scrogin KE. Sympathetic innervation of the splanchnic region mediates the beneficial hemodynamic effects of 8-OH-DPAT in hemorrhagic shock. Am J Physiol Regul Integr Comp Physiol 303: R527–R538, 2012. First published June 20, 2012; doi:10.1152/ajpregu.00689.2011.—Administration of the 5-HT1A receptor agonist, 8-OH-DPAT, improves cardiovascular hemodynamics and tissue oxygenation in conscious rats subjected to hypovolemic shock. This effect is mediated by sympathetic-dependent increases in venous tone. To determine the role of splanchnic nerves in this response, effects of 8-OH-DPAT (30 nmol/kg iv) were measured following fixed-arterial blood pressure hemorrhagic shock (i.e., maintenance of 50 mmHg arterial pressure for 25 min) in rats subjected to bilateral splanchnic nerve denervation (SD). Splanchnic denervation decreased baseline venous tone as measured by mean circulatory filling pressure (MCFP) and accelerated the onset of hypotension during blood loss. Splanchnic denervation did not affect the immediate pressor effect of 8-OH-DPAT but did reverse the drug’s lasting pressor effect, as well as its ability to increase MCFP and improve metabolic acidosis. Like SD, adrenal demedullation (ADMX) lowered baseline MCFP and accelerated the hypotensive response to blood withdrawal but also reduced the volume of blood withdrawal required to maintain arterial blood pressure at 50 mmHg. 8-OH-DPAT raised MCFP early after administration in ADMX rats, but the response did not persist throughout the posthemorrhage period. In a fixed-volume hemorrhage model, 8-OH-DPAT continued to raise blood pressure in ADMX rats. However, it produced only a transient and variable rise in MCFP compared with sham-operated animals. The data indicate that 8-OH-DPAT increases venoconstriction and improves acid-base balance in hypovolemic rats through activation of splanchnic nerves. This effect is due, in part, to activation of the adrenal medulla.

adrenal medulla; catecholamine; blood volume; mean circulatory filling pressure; venous tone

CURRENT METHODS OF RESUSCITATION from hypovolemic shock include rapid volume resuscitation with crystalloids, blood substitutes, or blood components. When volume resuscitation alone is insufficient to sustain peripheral perfusion, vasoconstrictors are administered (12). However, the use of directly acting vasoconstrictor agents prior to sufficient volume restitution can increase arterial resistance and exacerbate tissue ischemia. Ischemic tissue can, in turn, release circulating mediators that precipitate more severe inflammatory responses and multiorgan dysfunction or failure upon reperfusion (7, 23). Interventions that improve perfusion pressure through reductions in venous capacitance without significant increases in arterial resistance should produce less exacerbation of tissue ischemia and improve oxygen delivery, avert vascular dysfunction, and increase the probability of survival. Presently, few if any selective vasoconstrictor agents have been identified. We have shown that the 5-HT1A-receptor agonist, 8-OH-DPAT produces a sustained rise in arterial pressure among rats subjected to hemorrhagic shock (28–30). The pressor effect of the drug is paralleled by a sympathetically mediated increase in venous tone [as measured by mean circulatory filling pressure (MCFP)] and venous return (as measured by ascending aortic flow). However, 8-OH-DPAT does not raise total peripheral resistance, despite its pronounced sympathoexcitatory effect in hypovolemic rats. The rise in MCFP produced by 8-OH-DPAT is paralleled by improved acid-base balance and tissue oxygenation. Together, the data suggest that 8-OH-DPAT stimulates a preferential constriction of the venous vasculature, which, in turn, permits an advantageous redistribution of blood volume to the arterial side of the vasculature.

In the euvolemic mammals, the splanchnic vasculature contains ~33% of total blood volume (10). Much of this volume is contained within the venous vasculature, and because of the high compliance of venous vessels, a relatively large fraction of splanchnic blood volume does not contribute to transmural pressure in the central venous system under resting conditions, and, as such, is considered unstressed blood volume. Increased sympathetic activity directed to the splanchnic vasculature can dramatically reduce the capacitance of venous vessels, which results in mobilization of up to 60% of blood contained in the splanchnic venous vasculature. During blood loss, constriction of the splanchnic vasculature contributes to a significant redistribution of blood volume, in part, through diversion of blood away from the splanchnic arterioles and active constriction of splanchnic veins (26). The splanchnic vasculature is somewhat unique in its profile of sensitivity to vasoactive factors. Specifically, splanchnic arterioles are particularly responsive to the vasoactive peptides vasopressin and ANG II, but less responsive to sympathetic activity (31). In contrast, the majority of splanchnic vasoconstriction during hemorrhage is derived from sympathetic activity, with vasopressin having virtually no effect, and circulating ANG II providing only a minor contribution (26, 31). Given our evidence that the beneficial effects of 8-OH-DPAT on peripheral tissue perfusion is dependent upon sympathetic activation, and evidence in the literature indicating that sympathetic activation produces preferential constriction of venous splanchnic vasculature, we hypothesized that 8-OH-DPAT raises venous tone through sympathetically mediated splanchnic vasoconstriction. To date, there is no known means to produce selective vasoconstriction without also stimulating significant arterial vascular resistance and potential ischemia. Use of catecholamines to increase cardiac output can be dangerous in the absence of blood volume restitution. Here, we test the possibility that sympathetic acti-
vation of the splanchic vasculature may provide a means to improve hemodynamic compensation following hemorrhage, even in the absence of volume restitution. Specifically, we studied the effects of splanchic denervation on the hemodynamic effects of 8-OH-DPAT in rats subjected to hemorrhagic shock. Since splanchic sympathetic nerves also innervate the adrenal medulla, we further examined the effects of adrenal demedullation (ADMX) on the hemodynamic responses to 8-OH-DPAT in hemorrhaged animals.

METHODS

Animals
Male Sprague-Dawley rats weighing 320 to 380 g (Harlan, Indianapolis, IN) were maintained under standard conditions (22°C ambient temperature, 12:12-h light-dark cycle) in the institutional animal facility, with food and water available ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee. All experiments were conducted in compliance with the Principles of Laboratory Animal Care, as adopted and promulgated by the National Institutes of Health.

Surgery
Splanchic sympathetic denervation. Eighteen days prior to the experiment, rats were anesthetized with ketamine (100 mg/kg im) and xylazine (7 mg/kg im). The greater and lesser splanchic nerves were exposed bilaterally through a midline laparotomy and left intact or bisected, as described by others (14). In a separate pilot study, we found that acute denervation using this surgical procedure reduced increases in mesenteric vascular resistance during stimulation of the sympathetic fiber bundle proximal to the celiac ganglion by 90% in rats anesthetized with thiobutobarbitral (data not shown). The length of surgical recovery was chosen to enable rats subjected to denervation to regain sufficient body weight to match that of sham-operated controls at the time of the experiment, while avoiding significant reinnervation. In prior studies, celiac ganglionectomy was shown to produce profound loss of norepinephrine (NE) content in spleen and liver, and partial depletion (>50%) of the duodenum 2 wk after surgery. Observable reinnervation of mesenteric vascular was not observed until ∼5 wk after denervation (16).

ADMX. Seventeen days prior to experiment, rats were anesthetized with ketamine/xylazine anesthesia, as described above. The adrenal glands were exposed through bilateral 1.5-cm-long incisions along the dorsolateral border of the most caudal costal bones. The adrenal cortex was incised, and the medullary content was extracted by gentle pressure. Sham-operated animals were subjected to the same procedure, but the adrenal medulla was not expelled. Adrenal demedullation that is performed as described results in complete loss of osmotic stress-induced circulating epinephrine, but not corticosterone (2).

Vascular catheter implantation. Three days prior to the experiment, animals were anesthetized with pentobarbital sodium (60 mg/kg ip) for implantation of bilateral arterial femoral vascular catheters, and an inflatable balloon-tipped catheter (Vesta, Franklin, WI), as described previously (29). The balloon catheter was advanced into the right atrium via the left jugular vein to enable measurement of MCFP. A Silastic catheter (0.037 in. O.D.) was inserted into the femoral vein and advanced to the thoracic vena cava for measurement of central venous pressure (CVP). All catheters were flushed daily with 150 µl of saline containing 75 U/ml of heparin. The balloon catheter was inflated daily with 200 µl of saline to prevent fibrous accumulations around the catheter tip.

Experimental Protocols
Fixed-arterial blood pressure hemorrhage. Hemorrhage was initiated by withdrawing blood from one of the arterial catheters at the rate of 3.2 ml·kg⁻¹·min⁻¹ for 6 min, after which blood withdrawal continued for another 4 min at a reduced rate of 0.53 ml·kg⁻¹·min⁻¹. Over the next 15 min, mean arterial pressure (MAP) was maintained at 50 mmHg by manual withdrawal of additional small aliquots of arterial blood (∼150–200 µl) every few minutes. Twenty-five minutes after initiation of hemorrhage, blood withdrawal was terminated, after which drug or vehicle was delivered as described in the specific protocols outlined below. All withdrawn blood was collected and weighed for determination of total blood volume withdrawal.

Fixed-volume hemorrhage. In a subset of experiments, hemorrhage was initiated while the volume of blood withdrawal was strictly controlled. This enabled comparisons of MCFP between groups when one group was subjected to an intervention that impaired compensatory responses. During the first 10 min of hemorrhage, blood was withdrawn by a syringe pump as described above in the controlled pressure model. An additional 13 ml/kg of blood was removed in five aliquots. Two aliquots of 2 ml/kg were taken 12 and 15 min after initiation of hemorrhage. Subsequently, three aliquots of 3 ml/kg each were taken at 18, 21, and 24 min of hemorrhage period. Each aliquot was removed over a 25- to 35-s period.

Blood volume measurement. In a subset of animals, blood volume measurements were performed using the Evans blue dye (EBD) dilution method (17). Prior to the initiation of hemorrhage, a 0.3-ml sample of arterial blood was collected for use as a blank. Immediately afterward, 100 µl of EBD (5 mg/ml in saline) was injected into the venous catheter and flushed with 200 µl of isotonic saline. Ten minutes after EBD injection, 150 µl of blood was withdrawn from an arterial catheter. The sample was centrifuged for determination of hematocrit (Ht). Fifty microliters of plasma was diluted 1:20 in 0.1 M Tris-HCl buffer (pH 9.0). The absorbance of the sample and blank was determined at 620 nm (A620). A standard curve was constructed by linear regression analysis of the A620 values for a range of EBD concentrations (0.01–0.2 mg/ml) diluted as above with normal rat plasma.

Plasma volume (PLV) was calculated as PLV = (Ct × Vi)/Cp, where Ct and Vi were the concentration and volume of EBD injected and Cp was the plasma EBD concentration determined from the standard curve. Thus, blood volume was calculated as follows: BV = PLV/(1 − Ht). A second blood volume measurement was performed at the end of experiment using the same protocol.

Determination of tissue norepinephrine by HPLC. The extent of denervation was determined by measuring NE concentrations in spleen, liver, and small intestine using HPLC with electrochemical detection. Extraction of catecholamines was performed, as described previously (8). Tissue samples weighing between 120 and 160 mg were diluted to a final volume of 1 ml with 0.2 M perchloric acid (PCA). Samples were homogenized on ice for 10 min after the addition of 50 µl of 10% EDTA and 50 µl of isoproterenol (5 µM in 0.1 N HCl). After centrifugation, a 550-µl aliquot of supernatant was added to 25 mg of activated alumina (GFS Chemicals, Columbus, OH), 25 µl of 1.5% reduced glutathione, 25 µl of 10% EDTA, and 500 µl of 1 M Tris-buffer (pH 8.6), and mixed for 30 min at 4°C. The supernatant was aspirated, and the alumina was washed 3 times with 1 ml of 2.7 mM EDTA. Catecholamines were then eluted with 200 µl of 0.2 M PCA. The eluate (150 µl) was stored at −80°C until analysis. Recovery of an internal standard (isoproterenol) was 43–68%. Tissue pellets were resuspended in 1.5 ml of 0.1 N NaOH for protein determination by BCA protein assay (Thermo Scientific, Rockford, IL).

Norepinephrine was assayed by HPLC-ECD using an Eicom HTEC-500 system equipped with an Eicompak SC-3ODS column (Eicom, Kyom, Japan). The mobile phase consisted of 80% citrate-acetate buffer (pH 3.5), 20% methanol, including 220 mg/l of sodium octane sulfonate and 5 mg/l of EDTA-2 Na. The flow rate was set to 320 µl/min and the working electrode potential was set to +750 mV relative to an Ag/AgCl reference electrode. Chromatograms were...
collected and analyzed using PowerChrom software (Eicom, Kyoto, Japan). NE was quantified by determination of peak area, corrected for recovery and compared with standard curves (1–250 nM). The values were normalized to protein content and expressed as picogram per milligram protein.

Protocol 1. Effect of 8-OH-DPAT on splanchic nerve-denervated rats subjected to fixed arterial blood pressure hemorrhage. On the day of experiment, unanesthetized splanchic nerve denervated (SD) rats (n = 19) and sham-operated (Sham, n = 19) animals were connected to the recording instruments and allowed to habituate for at least 30 min before two baseline measurements of MCFP were taken 20 and 10 min prior to initiation of hemorrhage. A fixed arterial blood pressure hemorrhage protocol was initiated as described above and continued for 25 min. Both, SD- and Sham-operated rats, were randomly assigned to receive either a bolus injection of isotonic saline (200 μl iv) or 30 nmol/kg iv of 8-OH-DPAT at the end of blood withdrawal. After treatment, rats were monitored for an additional 35 min. Arterial blood pressure, HR, and CVP were recorded continuously throughout the experiment. Measurements of MCFP were performed 20, 30, 40, 50, and 60 min after initiation of hemorrhage. Samples of arterial and venous blood (150 μl) were withdrawn just prior to hemorrhage, at the end of hemorrhage (25 min), and at the end of the experiment (60 min) for determination of blood gases and acid-base balance. Animals were subsequently euthanized with pentobarbital sodium (100 mg/kg iv). Immediately after euthanasia, the spleen, frontal lobe of the liver, and a 10-cm section of the jejunum were collected from each rat, rapidly frozen in liquid nitrogen, and stored at −80°C until assayed for norepinephrine content.

Protocol 2. Effect of 8-OH-DPAT on rats subjected to ADMX and fixed-arterial pressure hemorrhage. Fourteen rats subjected to prior ADMX were exposed to fixed arterial blood pressure hemorrhage, as described above. Rats were randomly assigned to receive either bolus intravenous injection of a 200 μl of isotonic saline or 30 nmol/kg of 8-OH-DPAT. Hemorrhage and cardiovascular measures were carried out as described above in protocol 1.

Protocol 3. Effect of 8-OH-DPAT on rats subjected to ADMX and fixed-volume hemorrhage. On the day of experiment, unanesthetized ADMX (n = 6) and sham-operated (n = 6) rats were connected to the recording instruments and habituated for at least 30 min, after which determinations of blood volume was performed as described above. Fixed-volume hemorrhage was initiated 1 min after completion of the baseline blood volume measurement. Immediately after hemorrhage termination, a 30 nmol/kg dose of 8-OH-DPAT in 200 μl of isotonic saline was administered intravenously to all rats. Arterial blood pressure, HR, and CVP were measured continuously, as described above. The post-hemorrhage monitoring period continued for 35 min, after which a second blood volume measurement was performed. At the end of experiment, all rats were euthanized with intravenous injection of pentobarbital sodium (100 mg/kg iv).

Data acquisition and analysis. Arterial blood pressure and CVP were recorded on a Macintosh G4 PowerBook computer using PowerLab data acquisition software (Chart V5.2.1.; ADInstruments, Grand Junction, CO). Heart rate was calculated online using peak-to-peak detection of the arterial pulse pressure wave. MCFP was measured using methods described by Yamamoto et al. (33). Briefly, the right atrial balloon was rapidly inflated with 300–350 μl of saline to produce a brief (5–7 s) circulatory arrest. During the procedure, MAP falls and stabilizes at a nadir (final arterial pressure, FAP) while CVP rises and stabilizes at nearly the same level (venous plateau pressure, VPP). Mean circulatory filling pressure is then calculated using the following equation: MCFP = VPP + 1/60 (FAP − VPP). The 1/60 factor accounts for the difference in compliance between the arterial and venous vasculature in the rat. Measurements of VPP and FAP were taken once hemodynamic equilibrium was reached, usually, within 5 s of circulatory arrest, prior to activation of sympathetic reflexes (30). Total blood withdrawn was weighed to obtain a measure of total loss assuming a blood density of 1 g/ml. Blood gases were determined using an i-STAT 1 Analyzer (i-STAT, East Windsor, NJ). Plasma protein concentrations were determined using a handheld clinical refractometer.

Appropriate three- and two-way ANOVAs with repeated measures were used to determine effects of SD or ADMX on MAP, HR, CVP, and MCFP over time during fixed-blood pressure or fixed volume hemorrhage. Bonferroni (Dunn) post hoc tests were used to determine group differences at time points shown by symbols (i.e., every minute for the first 10 min, then every 5 min until drug injection). Within-group post hoc comparisons using Dunnett’s tests were used to determine the time at which variables differed from baseline during the course of hemorrhage. Student’s t-tests were used to determine the effect of SD and ADMX on baseline MCFP. Three and two-way ANOVAs with repeated measures were used to assess effects of 8-OH-DPAT and SD or ADMX on MAP, HR, CVP, MCFP and blood gases over time after termination of fixed-blood pressure hemorrhage (between 30 and 60 min after termination of blood withdrawal).

Fig. 1. Mean arterial pressure (MAP; top), heart rate (HR; middle) and central venous pressure (CVP; bottom) during hemorrhage and subsequent treatment with 8-OH-DPAT or saline (arrow) in splanchic-denervated (SD) and sham-operated (Sham) rats. Data are expressed as means ± SE. Filled symbols indicate no difference from baseline within group during hemorrhage (gray-shaded region). Open symbols indicate within-group difference from baseline (P < 0.05) during hemorrhage. §§P < 0.01 between groups; ***P < 0.01, Sham–8-OH-DPAT vs. Sham–Saline; #P < 0.05, SD-8-OH-DPAT vs. SD–Saline; ++P < 0.01 Sham–8-OH-DPAT vs. SD-8-OH-DPAT.
Table 1. Body weight, total blood loss, hematocrit and plasma protein concentration at baseline, after termination of hemorrhage (prior to subsequent treatment with saline or 8-OH-DPAT; 60 min) in splanchic-denervated, sham-operated, and adrenal demedullated rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>BW, g</th>
<th>TBL, ml/kg</th>
<th>Hematocrit, %</th>
<th>Plasma Protein, g/dl</th>
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<tr>
<td></td>
<td></td>
<td>BL</td>
<td>25 min</td>
<td>60 min</td>
<td>BL</td>
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<tr>
<td>SD-Saline</td>
<td>9</td>
<td>348 ± 4</td>
<td>33.9 ± 0.6</td>
<td>29 ± 1**</td>
<td>6.5 ± 1</td>
</tr>
<tr>
<td>SD-8-OH-DPAT</td>
<td>10</td>
<td>46 ± 1</td>
<td>31 ± 1**</td>
<td>27 ± 1**++</td>
<td>6.5 ± 0.1</td>
</tr>
<tr>
<td>Sham-Saline</td>
<td>9</td>
<td>350 ± 5</td>
<td>35.0 ± 0.4</td>
<td>32 ± 2**</td>
<td>6.4 ± 0.2</td>
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<tr>
<td>Sham-8-OH-DPAT</td>
<td>10</td>
<td>49 ± 1</td>
<td>33 ± 1**</td>
<td>30 ± 1**</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>ADMX-Saline</td>
<td>7</td>
<td>352 ± 7</td>
<td>31 ± 0.9##</td>
<td>33 ± 1**</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td>ADMX-8-OH-DPAT</td>
<td>7</td>
<td>46 ± 1</td>
<td>31 ± 1**</td>
<td>29 ± 1**</td>
<td>6.4 ± 0.1</td>
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</table>

Data are group means ± SE. BW, body weight; TBL, total blood loss; SD, splanchic-denervated; ADMX, adrenal demedullated. **P < 0.01 vs. baseline value (BL); +P < 0.05, vs. value at 25 min; ++P < 0.01, vs. value at 25 min; ##P < 0.01 vs. sham (pooled).

Significant main effects and interactions between factors were followed up with Bonferroni (Dunn) post hoc analyses. However, an a priori decision was made to perform group pair-wise comparisons of blood gases at 60 min. Separate Student’s t-tests were used to determine effects of SD on NE tissue levels. Ht and plasma protein concentration were compared between groups using appropriate two- and three-way ANOVA with repeated measures. P values of <0.05 were considered significant.

RESULTS

Effect of Splanchnic Denervation on Cardiovascular Response to Hemorrhage

There was no effect of randomization to drug treatment group prior to actual drug administration. Therefore, data representing cardiovascular parameters during active hemorrhage were pooled across SD and sham-operated groups. In intact rats, MAP remained stable for the first 3.5 min of blood withdrawal, then fell precipitously from the start of hemorrhage and tended to level off as the withdrawal rate was reduced 6 min after the start of hemorrhage. Central venous pressure then fell more gradually from the start of hemorrhage and tended to level off as the withdrawal rate was reduced 6 min after the start of hemorrhage. Central venous pressure then continued to decline more slowly with additional blood withdrawal. Arterial pressure fell more rapidly in rats subjected to SD. Heart rate tended to fall more rapidly in rats subjected to SD, but the within-group variability obscured group differences. The CVP response to blood withdrawal did not differ significantly between groups. The total blood volume required to maintain pressure did not differ between SD and intact rats (Table 1).

Effect of 8-OH-DPAT on Cardiovascular Parameters After Splanchnic Denervation

Intact animals given saline after termination of blood withdrawal showed little change in MAP following termination of hemorrhage (Fig. 1). Animals subjected to SD and given saline in the posthemorrhage period maintained arterial pressure until the last 10–20 min of the recording period when MAP began to fall. However, the fall in pressure was not significant, nor was there a difference in MAP between SD and control rats given saline. Administration of 8-OH-DPAT caused an immediate increase in MAP in sham-operated rats. The pressor effect was well sustained until the end of the recording period. The initial pressor response to 8-OH-DPAT was only slightly reduced in rats subjected to SD. However, the pressor effect of the drug was not maintained, and MAP declined to levels below that of intact 8-OH-DPAT-treated rats within 15 min of drug administration. Neither HR nor CVP were significantly affected by drug administration.
period. It was noted that in sham-denervated, saline-treated rats, MCFP continued to fall after the termination of hemorrhage and reached a nadir at the 30-min measurement point. Therefore, additional follow-up tests were performed to examine effects of SD on MCFP in saline-treated rats after hemorrhage termination (30–60 min). A two-way ANOVA with repeated measures showed an interaction between surgical treatment and time on MCFP following termination of hemorrhage. Follow-up Dunnett’s test using the 30-min time point, as the starting value showed an increase in MCFP in sham-operated rats at the 50- and 60-min time points. The interaction was due to a tendency for MCFP to fall in SD rats over time, while it rose over time in sham-operated rats.

Regression analysis of MAP and MCFP at 5, 15, 25, and 35 min after drug or saline administration showed significant correlations when all groups were included (Fig. 3). However, in the two earlier time points, the significance of the overall correlation was due to the separation of the groups with the intact 8-OH-DPAT-treated group at one extreme and the other three groups falling to the other extreme. Within-group correlations tended to be flat with the exception of the sham-operated, saline-treated group, which consistently showed tendencies or significant correlations within group as recovery progressed ($P = 0.25$ at 30 min, $P = 0.018$ at 40 min, and $P = 0.011$ at 50 min). In addition, the slope of the relationship in this group always paralleled the overall correlation. In the later time points, the correlation also became significant in SD rats treated with 8-OH-DPAT. At the end of hemorrhage, MAP and MCFP were highly correlated when all groups were included ($P < 0.01$). Liner regression analyses of the individual groups were either significant (Sham–Saline, $P < 0.05$; SD-8-OH-DPAT, $P < 0.01$) or showed a strong tendency for significance (Sham–8-OH-DPAT, $P = 0.076$), with very similar slopes to the overall regression except for SD rats given saline, which showed no correlation between MAP and MCFP.

Effect of Splanchnic Denervation on 8-OH-DPAT-Induced Changes in Acid-Base Balance

Splanchnic denervation did not affect baseline blood pH or bicarbonate levels (Table 2). Twenty-five minutes after initiation of hemorrhage, both denervated and intact groups showed evidence of severe metabolic acidosis with secondary respiratory alkalosis (Fig. 4). Specifically, venous blood pH, base excess, and $\text{Pa}_2\text{CO}_3$ were reduced while lactate was increased. There was no effect of SD on the extent of metabolic acidosis by 25 min after the start of hemorrhage prior to drug intervention. Metabolic acidosis continued to worsen after termination of hemorrhage in SD rats, as well as in intact saline-treated rats, which showed further declines in base excess and venous pH, as well as further increases in lactate. Intervention with 8-OH-DPAT prevented a further decline in base excess and venous pH and reversed the increase in lactate in rats with intact splanchnic innervation. In contrast, SD treated with 8-OH-DPAT tended to show more pronounced metabolic acidosis (lower venous pH and $\text{HCO}_3^-$) than saline-treated SD rats, although these differences were not significant. Arterial $\text{PO}_2$ increased throughout hemorrhage and recovery in all groups, indicative of compensatory respiratory responses. Arterial $\text{PCO}_2$ levels continued to decline through recovery in SD rats and Sham-operated rats treated with saline. Sham-operated 8-OH-DPAT-treated rats showed a slight recovery from values determined 25 min after the start of hemorrhage, indicating a reduction in secondary respiratory alkalosis.

Effects of Splanchnic Denervation on Tissue NE Content and Spleen Contractility

Splanchnic denervation eliminated more than 90% of NE content in the spleen ($1,782 \pm 173$ vs. $153 \pm 18$ pg/mg protein). Denervation reduced NE content in jejunum samples by ~34%.
Table 2. Arterial and venous blood pH and base excess before, immediately after (25 min), and 35 min after (60 min) termination of hemorrhage in rats treated with saline or 8-OH-DPAT

<table>
<thead>
<tr>
<th></th>
<th>Arterial pH</th>
<th>Arterial HCO₃⁻</th>
<th>Venous pH</th>
<th>Venous HCO₃⁻</th>
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<tr>
<td>Sham-Saline (9)</td>
<td>7.49 ± 0.01</td>
<td>29.5 ± 0.5</td>
<td>7.46 ± 0.01</td>
<td>32.1 ± 0.4</td>
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<td>25'</td>
<td>7.38 ± 0.01</td>
<td>13.1 ± 1.88§§</td>
<td>7.22 ± 0.04§</td>
<td>19.5 ± 1.38§</td>
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<tr>
<td>60'</td>
<td>7.24 ± 0.14§§</td>
<td>10.8 ± 2.58§§</td>
<td>7.10 ± 0.08§§</td>
<td>15.1 ± 2.58§§</td>
</tr>
<tr>
<td>Sham-8-OH-DPAT (10)</td>
<td>7.48 ± 0.01</td>
<td>30.1 ± 0.5</td>
<td>7.46 ± 0.01</td>
<td>32.8 ± 0.4</td>
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<tr>
<td>25'</td>
<td>7.43 ± 0.01</td>
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<td>7.31 ± 0.04</td>
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<td>60'</td>
<td>7.42 ± 0.03</td>
<td>19.4 ± 2.18§§</td>
<td>7.35 ± 0.04**</td>
<td>23.2 ± 1.88§§</td>
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<tr>
<td>SD-Saline (9)</td>
<td>7.50 ± 0.01</td>
<td>28.9 ± 0.5</td>
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<td>7.41 ± 0.02</td>
<td>15.2 ± 2.38§</td>
<td>7.25 ± 0.05§</td>
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<td>60'</td>
<td>7.28 ± 0.08§</td>
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<td>SD-8-OH-DPAT (10)</td>
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<td>29.3 ± 0.4</td>
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<tr>
<td>25'</td>
<td>7.42 ± 0.02</td>
<td>12.9 ± 1.38§</td>
<td>7.22 ± 0.06§</td>
<td>18.3 ± 1.28§</td>
</tr>
<tr>
<td>60'</td>
<td>7.23 ± 0.08 +§§</td>
<td>7.04 ± 0.09###</td>
<td>7.41 ± 1.58###</td>
<td>12.7 ± 1.58###</td>
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</tbody>
</table>

Data are expressed as means ± SE. Group n indicated in parentheses. +P < 0.05 vs. 25' within group; **P < 0.01 vs. Sham-Saline; ###P < 0.01 vs. Sham; §§P < 0.05 vs. BL within group; §§§P < 0.01 vs. BL within group.

(70 ± 6 vs. 36 ± 4 pg/mg protein) and liver by about 50% (736 ± 58 vs. 485 ± 36 pg/mg protein).

Effect of Adrenal Demedullation on Hemodynamic Response to Hemorrhage and 8-OH-DPAT Administration

Adrenal demedullation accelerated the fall in blood pressure (P < 0.01) and HR (P < 0.01) during the initial period of blood withdrawal when the volume of blood withdrawal was fixed (Fig. 5). Central venous pressure did not differ between groups at any point during hemorrhage. Less blood volume withdrawal was required to maintain blood pressure during the fixed blood pressure portion of hemorrhage in rats subjected to ADMX (Table 1). Subsequently, blood pressure of saline-treated ADMX rats tended to recover during the posthemorrhage period, while that of intact saline-injected rats did not. 8-OH-DPAT produced a robust and persistent increase in MAP in rats subjected to ADMX that was comparable to that of intact rats. Neither HR or CVP were influenced by 8-OH-DPAT in either intact ADMX rats.

Effect of Adrenal Demedullation on MCFP Response to 8-OH-DPAT

Mean circulatory filling pressure can only be used as a relative index of venous tone when groups have comparable blood volume. Because rats subjected to ADMX had less blood volume removed during fixed blood pressure hemorrhage, assessment of MCFP was only compared in ADMX rats. 8-OH-DPAT, but not saline, produced an immediate increase in MCFP (Fig. 6). However, MCFP of saline-injected rats tended to recover after hemorrhage termination, which minimized group differences during the course of hemorrhage. As in the SD study, it was noted that MCFP continued to fall after termination of the hemorrhage in saline-treated rats and reached a nadir at the 30-min time point. To determine whether MCFP rose in saline-treated ADMX rats after this time point, a one-way ANOVA with follow up Dunnett’s test was performed and demonstrated significant increases in MCFP at the 40-, 50-, and 60-min time points. Regression analysis of MAP and MCFP at each time point of MCFP determination failed to show either overall or within-group correlations except 5 min after drug administration, at which time within-group values were clustered at opposite ends of the spectrum (data not shown).

Effect of Adrenal Demedullation on Circulating Blood Volume and Hemodynamic Response to 8-OH-DPAT After Fixed-Volume Hemorrhage

As observed in the previous experiment, ADMX accelerated the fall in blood pressure during active blood withdrawal (Fig. 7). A surgery × time interaction on heart rate was also observed (P < 0.05) due to a more rapid and severe decline in heart rate among rats subjected to ADMX, but group comparisons did not reveal a significant difference at any one time point. Adrenal demedullation did not affect CVP during active fixed-volume hemorrhage. 8-OH-DPAT produced a robust and persistent rise in blood pressure in both groups of rats. 8-OH-DPAT did not significantly affect either HR or CVP in ADMX rats.

ADMX significantly reduced baseline MCFP (P < 0.01, Fig. 8) but did not influence baseline blood volume (Table 3). 8-OH-DPAT produced a rapid and robust rise in MCFP in both ADMX and sham-operated groups subjected to fixed-volume hemorrhage. The effect dissipated in rats subjected to ADMX but persisted in intact rats. Regression analysis of MAP and MCFP showed no significant correlations until 35 min after 8-OH-DPAT administration, when all subjects were assessed together, r = 0.73, P < 0.05, and when ADMX saline-treated rats were assessed alone (r = 0.86 P < 0.05). Sham-ADMX rats treated with 8-OH-DPAT showed no correlation (r = 0.086) but were clustered together at the end of the MCFP/MAP spectrum. Neither blood volume nor measures of hemodilution differed between groups at the end of the recording period (Table 3).

DISCUSSION

In the present study, we showed that denervation of the thoracic splanchnic nerves prevented increases in MCFP following hemorrhage and prevented any observable correlations between MCFP and MAP. Denervation also reversed the prolonged pressor effect of 8-OH-DPAT, as well as the drug’s ability to increase MCFP and attenuate metabolic acidosis. Together, these findings indicate that the thoracic splanchnic nerves contribute to venoconstriction and blood pressure maintenance following hemorrhage and that the 5-HT₁A receptor agonist 8-OH-DPAT accelerates this effect. Although 8-OH-
DPAT continued to increase MCFP in the absence of adrenal medullary tissue, the response declined during the posthemorrhage recovery period. These findings strongly suggest that circulating catecholamines contribute to the venoconstrictor effect of 8-OH-DPAT, and the effect is most apparent later after drug administration. In contrast, the arterial pressor response to 8-OH-DPAT was not altered after ADMX. The observation that 8-OH-DPAT produces a full pressor response, but only a partial effect on MCFP in ADMX rats, suggests that arterial vascular resistance contributed to the pressor effect of 8-OH-DPAT in the absence of the adrenal medulla.

Splanchnic Nerve Control of Arterial Pressure During Hemorrhage

Denervation accelerated the fall in pressure during the initial phase of hemorrhage in conscious rats. Others have also shown that SD reduces the volume of blood withdrawal required to maintain pressure over the full 25 min of hemorrhage. ADMX also accelerated the blood pressure fall during hemorrhage, confirming evidence that circulating catecholamines help to maintain blood pressure during early blood loss (27). Thus, loss of splanchnic sympathetic innervation of the adrenal gland likely contributed to the accelerated fall in blood pressure during hemorrhage produced by SD.

In the current study, animals subjected to ADMX could not maintain blood pressure, as well as SD rats, during the later phase of the hemorrhage, as demonstrated by the smaller total volume of blood required to maintain arterial pressure in ADMX rats. It is not clear why SD, but not ADMX, animals were able to maintain arterial pressure after hypotension had been established, given that intact splanchnic nerves are required for hemorrhage-induced epinephrine release (27). One
possibility is that denervation supersensitivity may have developed in SD rats, but not ADMX rats. Recent work has shown that sympathetic denervation of the rat tail artery greatly increases the responsiveness of the isolated artery to ANG II and vasopressin by 2 wk after denervation (32). Both ANG II and vasopressin are significantly elevated during hypotensive hemorrhage and could conceivably provide a greater contribution to the maintenance of arterial pressure during prolonged hemorrhage in the denervated animal due to a similar denervation-induced supersensitivity to noncatecholamine vasopressor agents (13), although this possibility remains to be studied.

**Splanchnic Control of MCFP and Venous Tone**

Mean circulatory filling pressure is dependent upon both blood volume and venous tone. Although we did not measure blood volume in SD animals, others have shown that celiac ganglionectomy does not influence blood volume by 7 days after denervation (15). In the current study, we showed that ADMX itself did not affect blood volume either before or after hemorrhage. Therefore, it is reasonable to expect that initial blood volumes were not different prior to hemorrhage in SD and intact rats. Since blood volume withdrawal prior to drug administration was similar in SD and intact rats, we used MCFP as an index to compare the effects of 8-OH-DPAT on venous tone in SD and intact rats. Our findings that baseline MCFP was reduced in SD rats confirm prior evidence that splanchnic nerves support whole body venous tone in the anesthetized cat (5). In contrast, celiac ganglionectomy was shown to have little effect on baseline MCFP in conscious rats (15). In prior rat studies, MCFP values were slightly lower than values obtained from our intact conscious rats (–6.9 vs. 7.5 mmHg). Our findings that MCFP was also reduced by ADMX suggests that circulating catecholamines contribute to whole body venous tone in the euvolemic rat. In contrast, others have shown little effect of ADMX on resting baseline MCFP (18). Though our animals were given several days to recover from vascular catheter placement and were tested unrestrained in their home cage, basal differences in circulating catecholamines may have contributed to baseline differences in MCFP observed between our rats and those described in the literature.

As expected, hemorrhage greatly reduced MCFP in both SD and intact rats. However, there was no longer any group difference in MCFP 20 min after the start of hemorrhage. This was surprising given our evidence that this hemorrhage model demonstrates a significant and persistent secondary increase in sympathetic activity that tends to peak 25 min after the start of hemorrhage (30). We expected that the progressive rise in sympathetic activity would increase venoconstriction in intact animals, but not SD rats. It is possible that denervation supersensitivity in the venous vasculature masked our ability to observe an effect of splanchnic nerves on venoconstriction during active hemorrhage. Denervation supersensitivity to adrenergic agonists has been described in the splanchnic venous vasculature (3). In addition, splanchnic venous vessels express...
nerve blockade prevented the rise in MCFP produced by 8-OH-DPAT, with the large rise observed in sham-operated rats at the same MCFP 25 min after injection, the effect was minimal compared to SD rats treated with 8-OH-DPAT showed a slight rise in MCFP within the first 15 min of injection. Although nic denervation completely blocked the ability of 8-OH-DPAT to levels even lower than intact rats in the current study despite the fact that rats subjected to autonomic blockade in our prior study had less blood volume removed (29). Thus, sympathetic activity likely does contribute to MCFP during the course of hemorrhage in this model lending credence to the possibility that supersensitivity masked the effects of splanchnic nerves on venoconstriction during active hemorrhage.

Effects of 8-OH-DPAT on MCFP After Hemorrhage

8-OH-DPAT produced a rapid and persistent increase in MCFP that lasted at least 35 min after drug injection. Splanchnic denervation completely blocked the ability of 8-OH-DPAT to raise MCFP within the first 15 min of injection. Although SD rats treated with 8-OH-DPAT showed a slight rise in MCFP 25 min after injection, the effect was minimal compared with the large rise observed in sham-operated rats at the same time point. In a prior study, we also showed that ganglionic blockade prevented the rise in MCFP produced by 8-OH-DPAT after shock (29). Together, the data strongly suggest that sympathetic efferent projections of the thoracic splanchnic nerves mediate the increases in whole body venous tone produced by 8-OH-DPAT in hemorrhaged rats. However, our experimental design did not permit us to rule out a role for splanchnic sensory afferent nerves in the initiation of these reflex responses. This remains an intriguing possibility given our prior evidence that the sympathoexcitatory effect of 8-OH-DPAT is attenuated by sinoaortic nerve denervation (20). In fact, 8-OH-DPAT causes renal and lumbar sympathoinhibition when given to euvoelmic rats at doses comparable to those used here (4, 19, 25). As such, it is quite possible that 8-OH-DPAT modulates the effect of some undefined afferent signal that is normally silent or very minimal in euvoelmic animals. The origins of the signal remain to be determined.

Nevertheless, 8-OH-DPAT does stimulate the release of circulating catecholamines in euvoelmic animals. Therefore, we attempted to determine whether the effect of SD was mediated by loss of neural input to the adrenal medulla. Adrenal demedullated rats subjected to fixed blood pressure hemorrhage showed a pronounced increase in MCFP immediately after 8-OH-DPAT injection compared with ADMX rats treated with saline. The group difference dissipated within 10 min as the MCFP in saline-treated rats increased over the posthemorrhage period. Nevertheless, the immediate rise in MCFP in the 8-OH-DPAT-treated ADMX rats suggests that splanchnic nerves innervating vascular targets, rather than the aortic medulla, mediate the early rise in venous tone. However, we were unable to directly compare MCFP in ADMX and intact rats in the fixed arterial blood pressure experiment since the ADMX rats were subjected to less blood volume withdrawal. During fixed volume hemorrhage, 8-OH-DPAT produced a very rapid and similar magnitude increase in MCFP in both ADMX and intact rats. However, MCFP began to dissipate in ADMX animals, falling to levels above preinjection baseline, but below those of intact rats. Together, the results suggest that splanchnic innervation of both vascular targets and the adrenal medulla contributes to the increase in venous tone produced by 8-OH-DPAT. The neural input to the splanchnic vasculature appears to mediate the early response, while a combination of neural and hormonal venoconstriction contributes to the later effect.

Effects of 8-OH-DPAT on Arterial Blood Pressure After Hemorrhage

In prior studies, we found that the early pressor effect of 8-OH-DPAT was mediated by both a direct effect of the drug on α1-adrenergic receptors in the vasculature and a sympatheticic-dependent component most likely mediated by central 5-HT1A receptor activation (21). In the current study, 8-OH-DPAT produced an immediate increase in arterial pressure in both denervated and intact rats as expected, given the direct

Table 3. Body weight, blood volume, and hematocrit at baseline and after hemorrhage and subsequent treatment with 8-OH-DPAT, 60 min in ADMX, and sham-operated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>BW</th>
<th>BL</th>
<th>TBL</th>
<th>RBC loss</th>
<th>Ht</th>
<th>BL</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMX-8-OH-DPAT</td>
<td>343.3 ± 6.7</td>
<td>52.6 ± 1.9</td>
<td>30.9 ± 4.9</td>
<td>34.4 ± 0.2</td>
<td>62.6 ± 4.8</td>
<td>47.2 ± 0.8</td>
<td>30.5 ± 1.2</td>
</tr>
<tr>
<td>Sham-8-OH-DPAT</td>
<td>352.3 ± 4.4</td>
<td>50.0 ± 0.7</td>
<td>30.6 ± 2.0</td>
<td>33.6 ± 1.1</td>
<td>59.3 ± 4.0</td>
<td>46.8 ± 0.8</td>
<td>30.8 ± 1.5</td>
</tr>
</tbody>
</table>

Data are expressed as group means ± SE. Also shown are total blood loss (TBL) and red blood cell (RBC) loss from hemorrhage. BV, blood volume; Ht, hematocrit.
vascular effect of the drug. The pressor effect waned in SD rats, indicating the later pressor effect of 8-OH-DPAT was mediated by sympathetic nerves innervating the splanchnic region. It is not clear why the direct vascular effects of the drug are transient, while the sympathetic-mediated effects are more long-lived. In a prior study, we noted that 8-OH-DPAT produced a transient pressure rise early after administration in hemorrhaged rats subjected to autonomic ganglion blockade (29). These findings confirm our earlier hypothesis that the pressor effect of 8-OH-DPAT comprises a combination of direct vascular effects and sympathetic mediated effects, with the direct effects waning as the drug is cleared from the plasma. These findings further suggest that the centrally mediated sympathetic effects may persist longer than the direct peripheral effects, possibly due to differences in clearance of drug from the brain and plasma. Indeed, pharmacokinetic studies have demonstrated that 8-OH-DPAT achieves a much higher peak concentration in brain than in plasma and that brain concentrations remain higher longer than plasma concentrations (34).

Our prior results indicate that in the intact rat, 8-OH-DPAT raises blood pressure almost exclusively by increasing cardiac output (28). In accord, results from the current study suggest that the latter portion of pressor effect of 8-OH-DPAT is due to a relatively selective increase in venous tone in the splanchnic vasculature, which promotes a significant increase in venous return. In support of this view, we observed a highly significant correlation between the arterial pressure and MCFP at the termination of the experiment, 35 min after 8-OH-DPAT injection when all groups were included. Interestingly, the slopes of the relationship between MAP and MCFP in the individual groups were very similar to the overall slope, except for SD rats given saline, which showed no correlation. In contrast, within-group correlations between MAP and MCFP were not evident during earlier time points except for sham-operated, saline-treated rats, which showed either a trend or significant correlation after the first 30-min measurement of MCFP. These data suggest that splanchnic nerves are important in mediating venous return during compensation after hemorrhage termination. This view is further supported by evidence that MCFP continued to rise in the posthemorrhage period in sham–SD rats, but not in SD rats. Moreover, splanchnic nerves appear to be able to raise MCFP in the absence of circulating catecholamines as ADMX rats treated with saline showed a similar rise in MCFP over the posthemorrhage recovery period that was also correlated with MAP.

These data also demonstrate that different mechanisms mediate the early and late pressor effects of 8-OH-DPAT. For instance, SD rats showed a rapid increase in pressure in the absence of a rise in MCFP with 8-OH-DPAT administration, suggesting that direct arterial vasoconstriction contributed to the early pressor effect of the drug. In contrast, the significant correlation of MAP and MCFP among 8-OH-DPAT-treated SD rats after the initial pressor response had waned suggests that 8-OH-DPAT may still influence MCFP to a minor degree after denervation. Indeed, we noted that denervation of the greater and lesser splanchnic nerves did not completely deplete NE in the small intestine and liver, suggesting that some blood reserves in the splanchnic venous vasculature may have been responsive to 8-OH-DPAT. This may have accounted for the increases in MCFP noted late in the experiment in SD rats treated with 8-OH-DPAT, as well as the significant correlation between MAP and MCFP in this group late in hemorrhage recovery.

Overall, our data suggest that the delayed pressor effect of 8-OH-DPAT correlates highly with the MCFP, suggesting that increases in venous tone and venous return, rather than arterial resistance mediates the persistent arterial pressor response to 8-OH-DPAT in hypovolemic rats. This view is further supported by evidence that animals which were able to substantially increase MCFP in response to 8-OH-DPAT also had much better acid-base balance by the end of the experiment. Hemorrhage itself produced a pronounced metabolic acidosis (as indicated by decreased venous pH and increased lactate), as well as secondary respiratory alkalosis (demonstrated by reduced PaCO₂), while PaO₂ was similarly elevated in all groups. 8-OH-DPAT reversed the metabolic acidosis, but only in rats with intact splanchnic innervation. Together, these findings indicate that 8-OH-DPAT increased venous return (and thus cardiac output), which, in turn, improved delivery of oxygenated blood by stimulating splanchnic sympathetic drive. Our data further suggest that increased splanchnic nerve activation produced by 8-OH-DPAT promotes a preferential vasoconstriction. Indeed, others have demonstrated that the sympathetic contribution to splanchnic arterial resistance in hypovolemic shock is minimal compared with that of ANG II and vasopressin, while the venous splanchnic vasculature is much more sensitive to sympathetic input (31).

Contrary to this hypothesis, we observed that 8-OH-DPAT produced a persistent increase in arterial pressure among rats subjected to ADMX, even though the drug produced only a partial effect on MCFP. Thus, arterial resistance may have contributed to the persistent blood pressure effects of 8-OH-DPAT in ADMX rats. It is tempting to speculate that increases in circulating epinephrine stimulated by 8-OH-DPAT may normally limit increases in arterial vascular resistance produced by excessive vasoactive hormone release. In fact, β₂-adrenergic receptor agonist administration during hemorrhage lowers total peripheral resistance and increases right atrial filling pressure (1). Likewise, selective blockade of β₂-adrenergic receptors attenuates increases in cardiac output produced by infusion of epinephrine (11). At the same time, β₂-adrenergic receptor agonists have been shown to have little influence on MCFP during increased sympathetic drive, while nonselective β-adrenergic receptor agonists have been shown to produce only a modest decrease in MCFP in the presence of elevated sympathetic drive. Thus, the β₂-adrenergic agonist effects of epinephrine may have more impact on arterial vascular resistance than mean circulatory filling pressure. Moderate release of epinephrine from the adrenal gland might actually contribute to the beneficial hemodynamic response to 8-OH-DAPT by suppressing excessive arterial resistance. In the absence of the adrenal medulla, activation of the splanchnic nerves by 8-OH-DPAT could raise pressure, in part, through increases in arterial resistance though this remains to be determined. Nevertheless, our data showing that MAP and MCFP are correlated late into hemorrhage recovery treatment with 8-OH-DPAT in ADMX rats supports the view that at least part of the persistent pressor effect of 8-OH-DPAT is mediated by vasoconstriction that is independent of circulating catecholamines.
Perspectives and Significance

Our results demonstrate that the 5-HT_{1A} receptor agonist, 8-OH-DPAT, produces a hemodynamic pattern that is highly beneficial to the hypovolemic rat. The hemodynamic pattern is dependent on splanchnic innervation of both the vasculature, and the adrenal medulla and is sufficient to improve acid-base balance prior to volume resuscitation. Volume restitution itself can promote oxidative stress and tissue damage following severe ischemia (6). Our studies indicate that a 5-HT_{1A} receptor agonist accelerates a sympathetic reflex response that reduces tissue hypoxia and may, thereby, reduce the potential for oxidative radical formation upon resuscitation. However, the response to 8-OH-DPAT is dependent upon sympathetic innervation of the splanchnic region and presumably the presence of sufficient vascular reactivity to catecholamines. It remains to be determined whether 8-OH-DPAT or other 5-HT_{1A} receptor agonists will be useful as adjuvants to resuscitation. While studies herein suggest they may be beneficial during the compensatory phase of hemorrhage prior to fluid resuscitation, the dependence of drug action on intact sympathetic function suggests they may have little effect when given alone during the final decompensatory stage of hemorrhage. Nevertheless, they may be useful in combination with other adjuvants currently under development that are designed to improve vascular reactivity in the late stage of blood loss.

REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.L.T., K.P., and K.E.S. conception and design of research; R.L.T. performed experiments; R.L.T. and K.E.S. analyzed data; R.L.T., K.P., and K.E.S. interpreted results of experiments; R.L.T. and K.E.S. prepared figures; R.L.T. and K.E.S. drafted manuscript; R.L.T. and K.E.S. edited and revised manuscript; R.L.T., K.P., and K.E.S. approved final version of manuscript.

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