Evidence for a Vascular Sensitizing Factor in Plasma of Saline-Loaded Dogs

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SUMMARY This study investigates whether plasma extracts previously demonstrated to have natriuretic and antinatriferic activity have effects on vascular reactivity of rat cremaster arterioles. Plasma from hydropenic and saline-loaded dogs was subjected to Diafiltration, and eluted on a strong cation exchange column (SCX). The effects of intraarterial injections of various column fractions on constrictor responses to repeated injections of 33.3 ng of norepinephrine (NE) were used to indicate changes in vascular responsiveness in third order cremaster arterioles. SCX fraction I (void volume) from saline-loaded dogs (FI-S) caused an increase in constrictor response to NE of 101%. Increased vascular responsiveness peaked at 40 minutes and remained significantly elevated (p < 0.05) for 130 minutes. Fraction I from plasma of hydropenic dogs (FI-H) and fraction III from plasma of saline-loaded dogs (FIII-S) did not increase vascular responsiveness to NE. FI-S shifted the dose response curves for NE, arginine vasopressin, and angiotensin II parallel and to the left relative to control by a factor of 3.05-, 2.95-, and 5.63-fold, respectively, at the 50% constrictor dose. Systemic injections of FI-S, but not FI-H, caused a 10 mm Hg rise in blood pressure at 50 minutes, and blood pressure was significantly elevated for 30 to 90 minutes after injection (p < 0.01).

These data demonstrate a vascular-sensitizing factor in FI-S. The factor appears in the same chromatographic fraction previously demonstrated to contain natriuretic, antinatriferic, and digoxin-like activity. The correlation of these activities with salt loading suggests they are due to the same substance, which may be the putative natriuretic hormone. (Hypertension 4: 581-589, 1982)

KEY WORDS • natriuretic hormone • rat cremaster muscle • resistance vessels • vascular reactivity • norepinephrine • vasopressin • angiotensin II
formance liquid chromatography (HPLC) containing this factor cross react with antidigoxin antibodies and inhibit a purified hog brain Na,K-ATPase preparation, suggesting that NH may be an endogenous digoxin-like substance. Elevated concentrations of NH measured as endogenous digoxin-like immunoreactivity have been reported in African green vervets with two-kidney, one clip Goldblatt hypertension and in rhesus monkeys with spontaneous hypertension. However, in cross circulation studies in which blood from volume-expanded donor dogs caused natriuresis in recipient dogs, BP did not rise in the recipient animals. Thus it is not clear that NH is, in fact, a hypertensive producing factor.

If NH promotes or causes hypertension, extracts containing the factor from salt-loaded normotensive subjects should have some effect on vascular reactivity and BP in assay animals. Accordingly, the present study was designed to determine if plasma extracts, previously demonstrated to contain NH activity, had effects on vascular reactivity of the rat cremaster muscle arterioles. The anatomical features of the cremaster muscle allow direct visualization of its arteriolar bed. Third order arterioles were chosen for study because these are major resistance vessels in the cremaster muscle, and because major drops in pressure have been recorded in third order arterioles in the cheek pouch of hamsters with renovascular hypertension, and increased resistance has been found in the third-order arterioles of the spinotrapezius muscle of SHR. Presumably, any vascular sensitizing factor which also raises BP would have a major effect on these blood vessels.

Materials and Methods

**Rat Cremasteric Muscle Preparation**

Male Sprague Dawley rats (Harlan, Madison, Wisconsin) weighing between 100 and 200 g were anesthetized with 50 mg/kg intraperitoneal sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Illinois). Rats were placed on a heated, clear lucite board containing a pedestal near one end, and rectal temperature was maintained at 37°C. A tracheostomy was performed to maintain a patent airway, and the left carotid artery was cannulated for measurement of blood pressure (BP). Mean arterial blood pressure (MAP) was recorded as the electronic mean of the pulsatile pressure using a Grass recorder. Two tapered PE 10 catheters glued together were inserted into the left femoral artery to the level of the inferior epigastric artery, allowing either plasma extracts or known vasoconstrictors to be injected directly into the cremaster artery by way of the inferior epigastric artery. An infusion of 75 mM saline and Nembutal was begun through the left femoral vein thirty minutes after induction of anesthesia at a rate of 25 μL/min, and was calculated to deliver 0.40 μg Nembutal/g min. This dose of pentobarbital was shown in preliminary studies to maintain rats in a stable, lightly anesthetized state.

The left cremaster muscle was prepared for transillumination by a modification of the method developed by Baez. A ventral incision was made in the scrotal sac and the cremaster with its testicle was gently teased free from the scrotal sac and associated connective tissue. Using a cautery to control bleeding, the ventral aspect of the cremaster was cut from the caudal pole to the external inguinal ring. The testicle and connective tissue were cut free from the cremaster muscle. The muscle was pulled evenly over a glass topped pedestal with 0.35 mm surgical wire, and a coverslip was placed on top of the muscle.

Transillumination of the cremasteric muscle on a microscope stage allowed the microcirculation to be visualized with the aid of a television monitoring system (magnification 900 × ). Thirty to 60 minutes was allowed for equilibration of fluids between the coverslip and muscle, and the muscle was then inspected for impaired blood flow, petechiae, or other signs of damage. Vessels without clear visualization of the arteriolar walls were not used. Vascular diameter of third order arterioles was measured by a system using two caliper arms whose movements were electronically recorded on a Grass instrument, or an image splitting device after the method of Intaglietta and Tompkins. The latter electronically shifts the television image and records diameter changes on a Grass instrument. All arteriolar measurements were made of the internal diameter of the vessel. The third order arterioles were identified by branching pattern with the main cremaster artery being the first order arteriole, and each subsequent branch being sequentially numbered.

**Extract Preparation**

Plasma extracts were prepared by the method of Gruber and Buckalew. Female mongrel dogs were deprived of food and water for 12 hours, anesthetized with pentobarbital, and the trachea intubated. Volume expansion was produced by intravenous infusion of a volume of normal saline equivalent to 7% of body weight over a 2-hour period, as previously described. Hydrospenic dogs were handled in the same way except that saline was not given. Heparinized blood was collected from the jugular vein and centrifuged immediately at 4°C for 5 minutes at 5000 × g. The plasma was separated from the formed elements and allowed to incubate for thirty minutes at room temperature, a procedure previously shown to increase the amount of NH in the plasma. An equal volume of distilled water was then added to the plasma, which was acidified to pH 5.5 with 10% acetic acid and boiled for 20 minutes. After centrifugation for 20 min at 12,100 × g, the supernatant was stored at —65°C until further use. Supernatant equivalent to 10 ml of plasma was ultrafiltered through Diaflo UM10 membranes (Amicon Corp.) with a 10,000 Mr cutoff and the filtrate collected and lyophilized. The lyophylized powder was dissolved in 400 μL of 10% formic acid and applied to a Partisil SCX (strong cation exchange) HPLC column (Whatman Inc., Clifton, New Jersey) and eluted with a pyridine-acetate gradient (pH 3 to 4), as previously
Two fractions were collected: fraction I, the void volume, and fraction III, 30-50 min into the gradient. Hereafter, each fraction will be referred to as Fl and FlII, with the suffix S or H referring to samples from either salt loaded or hydropenic dogs, respectively.

**Time Course Study**

The length of time vascular reactivity was altered by any of the fractions was assessed by determining the response of vessel diameter to a bolus injection of NE every 10 minutes before and after injection of a plasma fraction. NE injections were given over a period of 1 sec by an automatic injector, and contained 33.3 ng of NE in 20 μl of lactated Ringer’s solution. After each NE injection, the change in arteriolar diameter was measured, and the percent change from resting diameter was recorded. During the control period, vessels responding to the standard dose of NE with less than 20% or a greater than 50% constrictor response were rejected because the response fell three standard deviations outside the normal response range determined in preliminary studies. Vessels were also rejected if the constrictor response to NE varied by more than 5% over the course of three control injection periods. Following the control period, a plasma fraction dissolved in 250 μl of lactated Ringer’s solution was injected over a five minute period. The sodium concentration of these extracts was approximately 3200 mEq/liter. After fraction injection, additional NE injections were continued for 180 minutes. In this study rats were pretreated with 10 mg/kg mecamylamine (Sigma Chemical Company, St. Louis, Missouri) to abolish any cardiovascular reflexes.

**Dose Response Curves**

To assess changes in vascular reactivity, and to gain some insight into possible mechanisms of action, dose response studies were performed with NE, angiotensin II (All), and arginine vasopressin (AVP). The preparation of the rat was modified for this study by having only one catheter with a dead space of 50 μl placed in the left femoral artery, and no catheter in the left carotid. The 50 μl catheter was used for injection of either a fraction or various concentrations of a vasoconstrictor agent. The vasoconstrictor agents in 30 μl aliquots were infused into the 50 μl catheter and flushed into the cremaster muscle with a 60 μl bolus of lactated Ringer’s solution. The cremaster was prepared as before, and third-order vessels selected for study. Vascular diameter changes for various concentrations of a vasoconstrictor were assessed as in the time course study.

Dose response curves for NE and AVP were made 35 min after injection of Fl-S, since the time course study had indicated that peak response occurred at 40 min (fig. 1). Control dose response curves were made in a separate group of rats 35 min after injection of vehicle. NE dose response curves were made both with and without mecamylamine. Mecamylamine pretreatment eliminated nervous system reflexes, and duplicated the physiological state of the rats in the time course study. AVP dose response curves were made without mecamylamine.

Because of rapidly developing tachyphylaxis to All at higher doses, the protocol for the dose response curve for All differed from that used for NE and AVP. Since higher doses of All were not used, a control and experimental dose response curve was possible in the same rat. Thus, an All curve was made before and then 35 minutes after injection of Fl-S in the same rat. The pre-Fl-S curve served as a control within the group of rats given Fl-S. In a second set of control studies, the same protocol was used except that vehicle rather than Fl-S was injected. The dose response curve obtained 35 minutes after injection of the vehicle served as a timed control for the dose response curve obtained after Fl-S injection. The rats in the All study were pretreated with mecamylamine to reduce the variability of the All constrictor response.

**BP Study**

To assess the effects of Fl-S and Fl-H on MABP, rats were prepared for pressure recordings by place-
ment of a PE 90 catheter in the femoral artery, and the cremaster was prepared as before for diameter measurements. Two tapered PE 10 catheters were placed in the femoral vein, one for infusion of the 75 mM saline-Nembutal solution and the second catheter was used for systemic injections of one of the fractions. When blood pressure and arteriole diameter were stable for 30 minutes, a fraction was injected over a 5-minute period, following which MABP and diameter measurements were continued for 160 minutes. In five rats, the effects of both FI-S and FI-H were tested sequentially. In two other rats, only FI-S or FI-H were tested.

Data Analysis
The time course and BP studies were first analyzed for statistical differences between experimental and control curves using two-way analysis of variance. In addition, Dunnett’s test,19,20 a multiple comparison procedure that compares several experimental points with their own control, was used to determine where differences existed within a curve. In some cases, Student’s t test was also used to assess differences at particular time intervals between hydropenic and saline samples.

The dose response curves were first subjected to analysis of variance (ANOVA) for repeated measures.21 This test showed whether curves following FI-S injections were different from curves following vehicle injections, and whether there was any interaction between the two curves. If there was no interaction, it was assumed that the two curves were parallel. In addition, Brownlee’s method22 was used to test for possible differences in slopes as a second indication of whether the two curves were parallel. The fifty percent constrictor dose (CD50) for each curve was calculated from the best fit using least squares methodology for each rat’s dose response curve and compiled to obtain mean and standard error of the mean.

Drug Preparation
Each of the drugs was made fresh each day by dilution from stock solutions. Norepinephrine (Arterenol, free base). AVP (Grade V, synthetic) and All (Isoleucine form) were obtained from Sigma Chemical Company (St. Louis, Missouri). Stock solutions of NE were made in 0.1 N HCl from powder and kept frozen until used. AVP was purchased in a stock solution of normal saline and 0.5% chlorbutol. All was made in 0.05 M acetic acid from powder in siliconized glassware and kept frozen in small aliquots until used. All stock solutions were diluted to desired concentrations in lactated Ringer’s solution.

Results
Time Course Study
Figure 1 shows the effect of FI-S and FI-H on vascular responsiveness to a bolus injection of NE. The control constrictor response was 36.5% ± 4.4% and the response increased to a peak of 73.2% ± 8.0% 40 minutes following the injection of FI-S. This represents an increase in vascular responsiveness to NE of 100.8%. Injection of FI-H samples did not change the constrictor response from a baseline of 34.3% ± 3.4% during the control period. Analysis of variance showed a significant difference between the FI-S and FI-H (p < 0.001). Dunnett’s analysis indicated significant differences with time in the response to NE following FI-S but not FI-H, and these differences are indicated by asterisks in the figure. Vascular responsiveness was significantly increased at 10 min after the injection of FI-S and remained elevated for 130 minutes. Comparison of the FI-S and FI-H curves at individual time points by Student’s t test showed that the constrictor response to NE was significantly elevated for 130 min. This is consistent with the findings from Dunnett’s test.

The vascular diameters of the third order vessels were analyzed to determine if FI-S and FI-H altered the baseline diameter. Baseline diameter measurements were determined before each bolus injection of NE. The diameter measurements during the control period for FI-S and FI-H were 20.4 ± 0.3 μm and 21.6 ± 0.8 μm respectively and were not different from one another by t test (p > 0.1). This indicates that the vessels under observation were statistically the same size before either fraction was given. Analysis of variance indicated no difference between vascular diameter after administration of FI-S and FI-H, and Dunnett’s test indicated no change in baseline diameters over time for either fraction from their respective control.

Figure 2. A. Effects of SCX FIH-S on third-order arteriolar responsiveness to 33.3 ng bolus injections of NE. B. Effects of 250 μl of a high sodium solution (3256 mEq/liter) on arteriolar responsiveness to 33.3 ng injections of NE. Neither FIH-S or the high salt solution caused an increase in vascular responsiveness.
Figure 3. Dose response curve for NE in rats pretreated with mecamylamine after intra-arterial injection of FI-S shows a significant parallel, leftward shift compared to control. As described for figure 1, a 100% change in diameter on the ordinate indicates complete closure of the arteriole. Each point represents the average response ± SEM to the various concentrations of NE on the abscissa.

Figure 4 shows the dose response curves for NE after intraarterial injection of FI-S in rats not pretreated with mecamylamine shows a significant parallel, leftward shift compared to control.
Blood Pressure Study

Figure 7 shows the effect of FI-S and FI-H on MABP. The baseline MAP rose from control values of 107 ± 1.9 mm Hg to 117 ± 3.9 mm Hg at 50 minutes. Analysis of variance showed a significant difference between FI-S and FI-H (p > 0.025). Dunnnett’s test showed a rise in BP at 30 minutes after the injection of FI-S, and the effect lasted through 90 minutes. No change in BP was seen in FI-H. The effect of systemic injection of FI-H and FI-S on third-order vessel diameter in the cremaster muscle was observed, and no change from control diameters was seen over the time course of the experiment by Dunnett’s test.

Discussion

We chose to study the effects of extracts containing the putative natriuretic hormone (NH) on the microcirculation rather than large conduit vessels, since peripheral resistance in both normotension and hypertension is determined by changes in diameter of these vessels. The present study demonstrates that plasma from saline loaded but otherwise normal dogs contains a factor that causes a prolonged increase in vascular reactivity of third order arterioles. The vascular sensitizing factor was found only in saline loaded dogs and in the same chromatographic fraction previously demonstrated to have NH-like activity.18

Although increased vascular reactivity due to an effect of FI-S was clearly shown in the time course studies, the mechanism was not apparent. It is unlikely that the vascular sensitizing factor is NE or NE-like, since it did not cause direct vasoconstriction and its effect was prolonged. Similarly, a direct vasoconstrictor effect would have been expected if FI-S caused release of NE from the synaptic terminals of the sympathetic nervous system. Furthermore, the vascular sensitizing effect of FI-S was not blocked by pretreatment of the assay preparation with mecamylamine. This indicates that the effect of FI-S is not due to sympathetic nervous system reflexes but rather suggests a direct effect at the level of the vascular smooth muscle.

If the sensitizing factor in FI-S caused a parallel leftward shift of the dose response curve for several agonists, it would suggest that the factor is a functional sensitizer which alters some component of the effector system common to those agonists. To test this possibility, dose response curves for NE, AVP, and All were performed following FI-S injections, and in each case, a parallel leftward shift was seen. These findings are most consistent with the concept that the factor in FI-S is a functional sensitizer. These studies do not indicate which step in the effector system is enhanced by the sensitizing factor. It is generally agreed that increased calcium concentration at the level of the myofibril elements in vascular smooth muscle is necessary for vasoconstriction.23, 24 In support of this concept, Goldberg et al.25 recently reported that the vasoconstrictor effect of NE, AVP, and All was prevented by the calcium.
transport blocker nifedipine. Thus, it is possible that the vascular sensitizing factor might somehow augment the effect of NE, AVP, and AII to increase intracellular calcium. These results are also compatible with the possibility that the vascular-sensitizing factor had some long acting effect on vasoconstrictor amine transport by sympathetic nerve terminals, a cocaine-like effect.

Although no direct vasoconstrictor effect of the factor in FI-S was found, the present studies should not be interpreted to indicate that the factor in FI-S has no such effect. It is possible that smaller arterioles or arterioles in a different bed might be more responsive to a direct vasoconstrictor effect, or that a larger dose of the factor might have a direct effect on the vessels used in the present study.

A rise in BP was seen when FI-S was injected systemically (fig. 7). The time course of the elevated BP was almost identical to the increase in vascular responsiveness to NE following FI-S injection into the cremaster muscle. This suggests that the rise in BP seen after FI-S injections is due to its effect to increase vascular reactivity of the microcirculation. However, these studies do not rule out a possible effect of FI-S on cardiac output, or a direct vasoconstrictor effect.

The observed rise in BP, though small, is significant. Most authors who have demonstrated transferable pressor factors have found it necessary to sensitize the assay animal first, either by total nephrectomy or by feeding 2% saline drinking water for 3 weeks prior to use. The assay rats in this study were given no special diet, and retained both kidneys. Thus, the ability to demonstrate even a small rise in BP in these studies is important.

Increased vascular reactivity is a generalized phenomenon observed in many studies of both human and experimental hypertension, the mechanisms of which have not yet been elucidated. Folkow et al. have suggested, on both experimental and theoretical grounds, that medial hypertrophy of the vascular smooth muscle could account for the increased vascular reactivity. However, increased reactivity to NE was found to precede the rise in BP in experimentally induced renal and renopral hypertension. In a possibly related situation, children of hypertensive parents showed increased reactivity to NE compared to children of normotensive parents. Furthermore, Hinke accounted for structural changes in the vascular wall and found that additional factors contribute to the increased reactivity seen for AVP and NE in DOCA-salt hypertension.

Several studies have suggested that some humoral factor or factors may be responsible for the increased vascular reactivity found in hypertension. Hansen and Bohr showed increased sensitivity to potassium, calcium, and epinephrine in isolated hindlimb arterial strips from DOCA-salt rats protected from the high wall stress of hypertension by a ligature. This methodology eliminated tonic neural input and suggested the presence of a vascular sensitizing humoral agent with a long lasting half life. Mizukoshi and Michelakis demonstrated the presence of a transferable, low-molecular-weight, long-acting factor that increased the reactivity seen for AVP and NE in DOCA-salt hypertension. Higher levels of the factor were found in malignant hypertensive subjects on a high salt compared to a low salt intake. Subsequently, these investigators reported similar observations in one-kidney, one clip hypertension in dogs and rats, and in dogs with one-kidney one-wrapped hypertension. Self et al. found a vascular sensitizing agent in the sera of rats who became hypertensive on a high salt diet. Barbour et al. recently reported a transferable vascular sensitizing factor in the sera of SHR, and of interest in view of the present studies. Bohlen found that third-order vessels in the cremaster muscle of the SHR were hyperresponsive to NE.

Several studies have implicated the putative NH in the pathophysiology of hypertension. Lesions of the anteroventral third ventricle area (AV3V) have been found to prevent the induction of hypertension or, in some cases, reduce the severity of established hypertension in several models, including one-kidney Grollman, two-kidney, one-clip, and DOCA-salt hypertension. The AV3V lesion also prevented release of NH and a vascular Na-K pump inhibitory factor following extracellular fluid volume expansion. Recent work

Figure 7. Effect of systemic injection of SCX Fl on mean blood pressure over a 160-minute time period is shown. The bar at time zero indicates the 5-minute injection period of the fraction, and each curve is comprised of six rats. Asterisks indicate significant changes from control by Dunnett's test. FI-S, but not FI-H, caused a significant change in blood pressure.
in our laboratory has indicated that NH cross reacts with anti-digoxin antibodies and inhibits Na,K-ATPase, and is therefore an endogenous digoxin-like substance (an “endoxin”). Subsequently, it has been shown that endoxin is elevated in African green vervets with two-kidney, one-clip hypertension, and in rhesus monkeys with spontaneous hypertension. Poston et al. have recently reported the presence of a circulating inhibitor of the ouabain sensitive 22 Na efflux of red and white cells in humans with essential hypertension. In addition, a circulating ouabain-like factor has been demonstrated in dogs with one-kidney one-wrapped hypertension, and in rats with reduced renal mass-saline hypertension. The present studies are the first to demonstrate directly that extracts containing the putative NH are capable of raising BP, and to indicate a mechanism whereby the pressor effect might occur.

The finding that NH raises BP is at variance with the studies of Rudd et al. in this laboratory who showed that the same extract used in the present studies (FI-S) causes natriuresis in water-loaded rats with no increase in BP. This apparent discrepancy may be due to at least two experimental differences in the way the two studies were conducted. First, in the study by Rudd et al., the rats weighed 192 to 300 g, whereas those in the present study weighed half as much (103 to 124 g). Thus, the larger rats may not have shown a pressor response because of the larger volume of distribution of the factor and the resultant lower plasma concentrations. Second, in the experiments by Rudd et al., the rats were water-loaded, which decreases circulating AVP levels. Thus, the increased reactivity due to FI-S might be offset by decreased levels of endogenous vasoconstrictors.

The finding that NH raises BP is also at variance with the cross circulation studies of Pearce et al. and de Wardener et al. who showed that recipient dogs receiving blood from volume-expanded donor dogs exhibited natriuresis but no blood pressure effects. However, Bahlmann et al. showed a 17 mm Hg rise in BP in a recipient dog, but interestingly, the pressor response was delayed in onset relative to the natriuretic response. These results taken together indicate that the natriuretic effect of NH is not dependent on an increase in BP, but do not rule out a pressor effect for this factor. The studies suggest that the pressor effect of NH occurs when NH concentrations exceed that level needed for the natriuretic response. This conclusion is consistent with the hypothesis that the kidney is the primary target organ for NH, with the BP effect being a second order phenomenon.

The implications of the present studies with regard to the role of NH in the pathophysiology of hypertension are of interest. The results suggest that chronic hypertension could not only result from elevated NH levels, but also from an interaction between normal levels of NH and subpressor elevations of other endogenous vasoconstrictors. In this regard, it is interesting to note that “subpressor” elevations of both AVP and NE have been found in some hypertensive subjects.

References

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