VR1-Mediated Depressor Effects During High-Salt Intake
Role of Anandamide

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Abstract—This study was designed to test the hypothesis that increased sensitivity of blood pressure to anandamide (AEA), an endocannabinoid compound, occurs during high-salt intake, which can be blocked by a selective vanilloid receptor 1 (VR1) antagonist, capsazepine (CAPZ). Intravenous administration of a metabolically stable analog, methanandamide (MethA), dose-dependently decreased mean arterial pressure (MAP) in conscious rats fed a high-sodium diet (HS) for 3 weeks but it had a minimal effect in normal sodium (NS)-treated rats. The MethA-induced decrease in MAP was significantly attenuated but not abolished by CAPZ, or a selective cannabinoid receptor 1 (CB1) antagonist, SR141716A, administered separately in HS-treated rats. The MethA-induced depressor effect was prevented by the combined administration of CAPZ and SR141716A in HS-treated rats. Likewise, administration of capsaicin, a selective VR1 receptor agonist, dose-dependently decreased MAP in both HS- and NS-treated rats. The depressor effect of capsaicin was more profound in HS-treated rats, which was prevented by CAPZ. Western blot showed that expression of VR1 but not CB1 in mesenteric arteries was increased in HS-treated compared with NS-treated rats. Therefore, these data show that: (1) HS upregulates mesenteric VR1 expression; (2) HS increases sensitivity of blood pressure to AEA; and (3) HS-induced enhancement of the depressor effect of AEA can be prevented only when both VR1 and CB1 receptors are blocked. These results indicate that AEA contributes to the prevention of salt induced increases in blood pressure via, at least in part, activating the VR1 receptor. (Hypertension. 2005;46[part 2]:986-991.)

Key Words: high salt intake ■ blood pressure ■ sensory nerves ■ neurotransmitters

Anandamide, isolated from porcine brain in 1992, is one of the endogenous ligands for the cannabinoid (CB) receptor. Cannabinoids are known to elicit neurobehavioral as well as cardiovascular effects. The biological effects of cannabinoids are mediated by specific receptors, of which 2 subtypes have been identified by molecular cloning. The CB1 receptors express abundantly in both the central nervous system and peripheral tissues, whereas CB2 receptor expression is essentially restricted to the immune system. Given their profound cardiovascular effects in humans and animals, there is ever-increasing interest in endogenous cannabinoids. Recent evidence shows that anandamide and its analogs cause a prolonged hypotension and bradycardia in anesthetized normotensive or spontaneously hypertensive rats. Although the hypotensive effect of anandamide may be mediated by the CB1 receptor in light of the fact that blood pressure response to anandamide can be blocked by the CB1 antagonist, SR141716A, the mechanism underlying the anandamide-mediated hypotension may be complex. We have shown that the methanandamide (MethA)-induced depressor effect in spontaneously hypertensive rats rats is attenuated by the vanilloid receptor 1 (VR1) antagonist, capsazepine (CAPZ), suggesting that activation of the VR1 receptor may be involved in anandamide-induced hypotensive effects.

Recently, several lines of evidence in vitro and in vivo have shown that the VR1 receptor may be involved in cardiovascular regulation induced by anandamide. VR1 is a ligand-gated nonselective ion channel located primarily in sensory nerves, including unmyelinated C-fibers or thinly myelinated Aδ-fibers. It is known that VR1 can be activated by a variety of physical and chemical stimuli including capsaicin and other vanilloid compounds. Sensory nerves densely innervate cardiovascular tissues including the heart and blood vessels. Activation of VR1 expressed in sensory neurons leads to release of a number of neurotransmitters, commonly calcitonin gene-related peptide (CGRP) and substance P, which are potent vasodilators in many vascular beds.

Extensive research on defining the relationship between salt and blood pressure demonstrates that a subpopulation of both normotensive and hypertensive subjects is sensitive to high salt intake. The study of salt sensitivity is therefore important in cardiovascular research, especially with respect to health disparities. Despite intensive investigation in this area, the molecular mechanisms underlying salt-induced increases in blood pressure are poorly defined. Although previous studies by us and others suggest that the VR1 receptor and sensory neurotransmitters may play a compen-
Theory in preventing blood pressure elevation in rats fed a high-sodium (HS) diet.\textsuperscript{11,12} The role of VR1 in salt-induced modulation of cardiovascular function and blood pressure is largely unknown, especially when considering our poor understanding about the endogenous agonists of this receptor. The objectives of this study were to determine: (1) whether anandamide, an endocannabinoid compound, induces a prolonged and dose-dependent hypotensive effect during HS intake and (2) if so, whether the depressor effect of anandamide in HS-treated rats is mediated by activation of the VR1 receptor.

Methods

Animals

All experiments were approved by the Institutional Animals Care and Use Committee. Experiments were performed using male Wistar rats (Charles River Laboratory, Wilmington, Mass). All rats (6 weeks old) were housed in the animal facility for 1 week before the experiments. They were then randomly assigned to a normal-sodium (NS) diet (0.4% of Na\textsuperscript{+} by weight, Harlan Teklad) or HS (4% of Na\textsuperscript{+} by weight; Harlan Teklad) for 3 weeks. All rats drank water ad libitum throughout the experiment.

Surgical Preparation

The rats were anesthetized with ketamine and xylazine (80 and 4 mg/kg intraperitoneally, respectively) for implantation of vascular catheters or with urethane (1.5 g/kg intraperitoneally) throughout protocol 2. The left jugular vein and carotid artery were cannulated under anesthesia for administration of drugs or monitoring of mean arterial pressure (MAP) and heart rate (HR) with a Statham 231D pressure transducer coupled to a Gould 2400s recorder (Gould Instruments).

Experimental Protocols

Protocol 1

Rats fed a NS or HS diet were randomly assigned to 5 groups for intravenous injection of vehicle or MethA alone or in combination with SR141716A (a CB1 receptor antagonist), CAPZ (a VR1 receptor antagonist), or the combination of the 2 (n=5 to 8). Baseline MAP and its response to the aforementioned drugs were determined 3 hours after surgery when animals were fully awake and unrestrained as described previously.\textsuperscript{7} MethA was administered in increasing doses (0.5, 5, and 15 mg/kg intravenous bolus) in 3 subgroups of rats (each group for each dose). To examine the effects of SR141716A and CAPZ on MethA-induced changes in MAP, MethA (5 mg/kg) was injected 20 minutes or 10 minutes after intravenous injection of SR141716A (3 mg/kg), CAPZ (3 mg/kg), or the combination of the 2.\textsuperscript{6,7,13} The doses and time frames for injection of these drugs were based on the results of our previous\textsuperscript{3} and current studies (see results) showing that SR141716A and CAPZ caused transient elevation in blood pressure in rats fed HS diet, which lasted for no more than 15 minutes and 7 minutes after injection of these drugs, respectively. Thus, MethA was injected when baseline MAP was restored and stable, which happened to be 20 minutes and 10 minutes after injection of SR141716A and CAPZ, respectively.

Protocol 2

To determine the role of VR1 as a depressor during HS intake, a selective VR1 receptor agonist, capsaicin (10 and 30 μg/kg bolus), or vehicle was intravenously injected into the anesthetized rats fed a NS or HS diet (n=5 to 6). Given that Capsaicin is an irritant and causes severe pain in conscious rats, this protocol was performed under in anesthetized rats. Each dose of injection was separated by a 30-minute interval. To determine the specificity of capsaicin, CAPZ (3 mg/kg) was administered 10 minutes before capsaicin injection in a separate group.

Protocol 3

Rats fed a NS or HS diet were euthanized by decapitation without subjecting to acute experiments. Mesenteric resistance arteries were collected for Western blot analysis of VR1 and CB1, and for immunohistochemical staining of VR1 and CGRP.

Western Blot Analysis

Membrane protein of the mesenteric arteries was extracted, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane as described previously.\textsuperscript{11} The membranes were blocked 1 hour at room temperature in 5% milk washing solution (50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1% Tween-20 at pH 7.5). Subsequently, the membranes were incubated with goat anti-rat VR1 polyclonal IgG (1:800; Santa Cruz Biotechnology) or goat anti-human CB1 polyclonal IgG (1:500; Santa Cruz Biotechnology) in blocking solution overnight at 4°C. After being washed, the membranes were incubated with bovine anti-goat IgG-HRP (1:2000; Santa Cruz Biotechnology) in blocking solution at room temperature for 1 hour. The membranes were developed using enhanced chemiluminescence ECL kit (Amersham Pharmacia Biotech) and exposed to film (Hyperfilm-ECL; Amersham Pharmacia Biotech). The films were scanned and analyzed by using the Image Quantity Program (Scion) to obtain integrated densitometric values. β-actin was used to normalize protein loaded on membranes.

Immunohistochemistry

Colocalization of CGRP and VR1 in mesenteric arteries were performed by confocal analysis of double-immunofluorescence staining and fluorescence microscope as described previously.\textsuperscript{11,14} Briefly, mesenteric arteries taken from rats fed a NS diet were fixed with Zamboni’s fixative solution for 4 hours at 4°C, and were incubated in 0.4% Triton-X 100 and 5% fetal bovine serum in phosphate-buffered saline for 1 hour at room temperature to block nonspecific binding. The arteries were then incubated with goat anti-rat VR1 polyclonal antibody (1:200; Santa Cruz Biotechnology) in phosphate-buffered saline with 5% fetal bovine serum overnight at 4°C, followed by incubation with rabbit anti-rat CGRP antiserum (1:400; Sigma) for 12 hours at 4°C. Subsequently, the vessels were incubated with Cy3-conjugated anti-rabbit IgG (1:500, Jackson ImmunoResearch) for 1 hour at room temperature. Finally, the vessels were incubated with fluorescein isothiocyanate (FITC-) conjugated anti-goat IgG (1:500; Jackson ImmunoResearch) for 1 hour at room temperature. The slides were viewed under Zeiss Pascal confocal laser scanning microscope using 488-nm and 543-nm laser. Negative control for possible cross-reactivity between the fluorescent reagents was performed by incubating the vessels with only one of the primary antibodies, followed by incubation with a mixture of secondary antibodies. No cross-reactivity was observed.

Drugs

Methanandamide (Sigma), capsaicin (Sigma), and SR141716A (provided by Dr Kaminski) were dissolved in ethanol (10% v/v), Tween-80 (10% v/v), and normal saline. Capsazepine (Calbiochem) was dissolved in dimethyl sulfoxide (10%; v/v), Tween-80 (10%, v/v), and normal saline.

Statistical Analysis

All values are expressed as mean±SE. Differences between 2 groups or before and after treatment were analyzed by using the unpaired or paired Student t test. The differences among groups were analyzed using 1-way ANOVA followed by a Bonferroni adjustment for multiple comparisons. Differences were considered statistically significant at P<0.05.

Results

There was no significant difference in body weight between 2 groups after 3-week dietary treatment (NS, 292±8 grams versus HS, 286±6 g, P>0.05). HS intake for 3 weeks...
increased baseline MAP when compared with rats fed a NS diet (114±3 mm Hg versus 105±3 mm Hg, *P*<0.05). MAP and HR responses to intravenous administration of MethA in rats fed a NS or HS diet are shown in Figure 1 and Figure 2. Administration of MethA caused a triphasic MAP response and a decrease in HR in both NS- and HS-treated rats. The initial transient hypotension was followed by a brief pressor and a prolonged depressor phase. The prolonged depressor phase 3 reached the peak 15 to 20 minutes after administration of MethA. The changes in MAP were associated with pronounced bradycardia. MethA led to a dose-dependent prolonged reduction in MAP in rats fed a HS diet. MethA (0.5, 5, 15 mg/kg) induced peak decreases in MAP by 6±2 mm Hg, 21±3 mm Hg, and 30±3 mm Hg in HS-treated rats, respectively, which were significantly greater than that found in NS-treated rats (4±2 mm Hg, 5±2 mm Hg, and 13±4 mm Hg, respectively). Particularly, MethA at 5 mg/kg has no effect in rats fed a NS diet, but it decreased MAP by 21±3 mm Hg in HS-treated rats (Figure 2A). Blockade of the CB1 or VR1 receptors with SR141716A or CAPZ significantly attenuated the prolonged depressor effect of MethA at this dose in HS-treated rats (9±1 and 11±2, respectively; Figure 2B). Moreover, combined administration of SR141716A and CAPZ prevented the MethA-induced depressor effect in HS-treated rats (4±1; Figure 2B). SR141716A, CAPZ, or the combination of the 2 did not affect the baseline MAP in rats fed a NS diet, but they significantly elevated MAP in rats fed a HS diet (15±3, 12±2, or 18±4 mm Hg, respectively) compared with that in rats fed a NS diet.

Effects of capsaicin, a selective VR1 receptor agonist, on MAP were studied in urethane-anesthetized rats (Figure 3 and Figure 4). Intravenous injection of capsaicin produced a triphasic MAP response similar to that caused by MethA in conscious rats. Capsaicin produced a dose-dependent depressor phase in rats fed a NS or HS diet. However, the magnitude of the decreases induced by capsaicin (10 and 30 μg/kg) was significantly greater in HS-treated rats compared with NS-treated rats (8±2 and 18±3 mm Hg in HS-treated rats versus 3±1 and 8±1 mm Hg in HS-treated rats). Moreover, the decreases in MAP were blocked by CAPZ in NS (4±1 and 5±1 mm Hg) and HS-treated (4±1 and 7±1 mm Hg) rats, values that were not significantly different from that in
vehicle-treated rats fed a NS (2±3 mm Hg) or HS (1±3 mm Hg) diet.

Western blot analysis was performed to determine the effect of HS intake on the protein expression of VR1 and CB1 in mesenteric arteries (Figure 5). HS treatment for 3 weeks increased VR1 receptor protein expression in mesenteric arteries when compared with rats fed a NS diet (HS, 0.41±0.03% of β-actin arbitrary versus NS, 0.28±0.03% of β-actin arbitrary; \(P<0.05\)). Although expression of the CB1 receptor in mesenteric arteries was undetectable, CB1 receptor expression in the renal cortex was increased by HS intake when compared with NS intake (HS, 0.2±0.01% of β-actin arbitrary versus NS, 0.14±0.01% of β-actin arbitrary; \(P<0.05\)), indicating that the lack of staining in the mesenteric arteries was not caused by technical problems.

Double staining of VR1 and CGRP, a marker of primary afferent fibers, revealed that VR1 receptors were expressed in perivascular sensory nerve fibers and colocalized with CGRP (Figure 6).

**Discussion**

The present studies were designed to characterize the action and mechanism of anandamide on blood pressure response to HS challenge. The major new findings are that: (1) HS upregulates mesenteric VR1 expression; (2) HS increases sensitivity of blood pressure to AEA; and (3) HS-induced enhancement of the depressor effect of AEA can be prevented only when both VR1 and CB1 receptors are blocked. Taken together, these findings indicate that MethA-induced prolonged hypertension is mediated by at least 2 different pathways during HS intake. One involves SR141716A-sensitive CB1 receptors, whereas the other is dependent on anandamide-activation of the VR1 receptors expressed in sensory nerves.

Although cannabinoids elicit prominent cardiovascular effects, relatively little is known about the mechanism of their effects.\(^{15,16}\) CB1 receptors are localized in the nucleus of the solitary tract (NTS) and in the dorsal motor nucleus of the vagus,\(^ {17,18}\) suggesting that CB1 expressed in the brain may be involved in the regulation of cardiovascular function. Although it has been shown that injection of cannabinoids or anandamide into medulla oblongata or NTS causes central sympatoexcitation via activation of the CB1 receptors,\(^ {19,20}\) evidence has been shown that cannabinoids presynaptically inhibit the release of noradrenaline from many postganglionic sympathetic neurons.\(^ {21–23}\) Therefore, cannabinoids may influence cardiovascular function via modulating autonomic outflow in both the central and peripheral nervous systems. Several studies have shown that the prolonged depressor phase 3, which follows the transient pressor phase, is attenuated by pretreatment with cervical spinal transection or cervical spinal transection.
α-adrenoceptor and cannabinoid receptor antagonists, indicating that the sympathetic nerve system may be involved in the depressor response. Moreover, it is evident that there is no centrally elicited effect on the firing rate of presympathetic sympahtoexcitatory neurons in the rostral ventrolateral medulla oblongata and of splanchnic sympathetic nerve fibers. These results suggest that peripheral actions appear to be predominate in cardiovascular regulation, at least on systemic administration at the doses used by most investigators.

In addition to CB1 receptor-mediated prejunctional inhibition of sympathetic outflow, cannabinoids have direct effects on the vasculature. The studies by Wagner and Vidrio et al have shown that the vasodilatory effects of the synthetic cannabinoids HU-210 and WIN-55212-2 are retained in sympathectomized rats receiving arginine vasopressin (AVP) and norepinephrine infusion. Also, the maximal hypotension induced by HU-210 exceeds that seen after α-adrenergic blockade, indicating that additional peripheral vasodilatory effects are present. Recent studies have focused on the potential mechanisms by which anandamide results in peripheral vasodilation. Anandamide may produce vasodilation by both endothelium-dependent, SR-141716A–sensitive, and endothelium-independent, SR-141716A–insensitive pathways. The documentation of CB1 receptor mRNA, anandamide, and the amidohydrolase which is responsible for anandamide metabolism in endothelial cells and renal tissues supports the concept of endothelium-dependent vasodilation. A potential mechanism for the endothelium-independent pathway may involve anandamide interaction with capsaicin-sensitive vanilloid receptors expressed in the perivascular sensory nerves causing release of CGRP and CGRP-mediated vasodilation.

Several studies report that anandamide displays a highly structural similarity to the vanilloids. The studies by Zygmunt et al demonstrate that anandamide and methanandamide act as agonists at the recombinant rat VR1 receptors. These findings are further confirmed by Smart et al using the human VR1 clone. Furthermore, Wagner et al have demonstrated that SR141716A, a selective CB1 receptor antagonist, fails to inhibit anandamide-induced vasodilation in endothelium-denuded mesenteric preparations from rats. This study suggests that anandamide-induced mesenteric vasodilation is partially mediated by an SR-141716A-resistant action on vascular smooth muscle. Zygmunt et al demonstrate that in isolated rat hepatic, rat mesenteric, and guinea pig basilar arterial preparations, anandamide-induced relaxation is almost completely blocked either by the selective VR1 receptor antagonist, CAPZ, or by the selective CGRP receptor antagonist, CGRP9-37, but not by SR141716A. These results suggest that the VR1-mediated release of CGRP from sensory nerves is responsible for the anandamide-evoked vasorelaxation.

Consistent with previous studies, our data show that CGRP-containing fibers innervate mesenteric arteries and co-express with VR1 receptors. It is well-established that CGRP is one of the most powerful vasodilator neurotransmitters. These observations suggest that the VR1-mediated CGRP release may participate in regulating peripheral vascular resistance under pathophysiological conditions. Our results show that an increased depressor response to capsaicin, a selective VR1 receptor agonist, occurs in rats fed a HS diet. These increased depressor effects of capsaicin may be caused by an upregulation of the VR1 receptor expressed in sensory nerves innervating the mesenteric arteries. The specificity of capsaicin is confirmed by the fact that CAPZ, a selective VR1 receptor antagonist, blocks the capsaicin-induced depressor effect. Consistent with these results, we have shown for the first time that MethA causes an enhanced and dose-dependent depressor effect in HS-treated rats that can be attenuated by CAPZ. These findings support the hypothesis that activation of VR1 receptors contributes to the hypotensive effect of MethA during HS intake.

The degree of CAPZ-induced attenuation of the depressor effect evoked by MethA is similar to that caused by SR141716A. In addition, combined use of CAPZ and SR141716A, but not application of either of these two compounds alone, prevents the MethA-induced depressor effect. These results indicate that there are 2 independent pathways mediating the MethA effect, ie, a SR141716A-sensitive, CB1-dependent pathway, and a CAPZ-sensitive, VR1-dependent pathway. The fact that total blockade of the MethA effect with the combined use of CAPZ and SR141716A indicates that residual depressor effects mediated by other mechanisms, if any, are negligible.

In conclusion, our present study demonstrates that anandamide produces a profound hypotension during HS intake and that the enhanced depressor effect of anandamide is mediated, at least in part, by activation of the up-regulated VR1 receptors during HS intake.

**Perspectives**

HS intake has been implicated in the pathogenesis of hypertension, particularly in salt-sensitive individuals. Studies in salt-dependent experimental animals and humans have shown that HS intake is associated with significant increases in blood pressure. Although animal models of salt-dependent hypertension have been developed, including genetic, pharmacological, and surgical methods, the mecha-
nistic link between dietary salt and hypertension remains poorly understood. Recently, several lines of evidence have shown that the sensory nervous system, including VR1 receptors expressed in sensory nerves and sensory neuro-transmitters, is involved in the regulation of blood pressure in salt-dependent hypertension.\(^\text{11,12}\) It is well-established that VR1 is activated by the plant extract capsaicin, but the identity of the endogenous mammalian ligands remains unclear. Given the fact that anandamide produces a depressor effect possibly via activation of the VR1 receptor in rats taking a HS diet, it is tempting to speculate that manipulations that modulate anandamide production or metabolism may alter VR1 function and therefore blood pressure. Therefore, our results may provide a rationale for the search of novel endogenous and/or exogenous VR1 activators for the treatment of salt-dependent hypertension and contribute to our understanding of an important issue in hypertension research, ie, health disparities.

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**References**

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