Anandamide-Induced Depressor Effect in Spontaneously Hypertensive Rats
Role of the Vanilloid Receptor
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Abstract—To test the hypothesis that activation of the vanilloid receptor (VR1) contributes to the anandamide-induced depressor effect in spontaneously hypertensive rats (SHR), we used a selective VR1 antagonist capsazepine (CAPZ) and a selective cannabinoid type 1 receptor antagonist SR141716A in conjunction with a VR1 agonist capsaicin in both SHR and Wistar-Kyoto rats (WKY). Mean arterial pressure was increased in SHR compared with WKY (P<0.05). Intravenous administration of capsaicin caused a greater depressor response in SHR compared with WKY (P<0.05), which was blocked by ≈60% by CAPZ (P<0.05) in SHR only. Methanandamide caused a similar greater depressor response (P<0.05), which was blocked by ≈50% and 60% by CAPZ and SR141716A, respectively, in SHR (P<0.05) but not in WKY. Radioimmunoassay showed that methanandamide increased plasma calcitonin gene-related peptide (CGRP) levels from baseline in both SHR and WKY (P<0.05), with no difference between 2 strains. Western blot showed that protein expression for the calcitonin receptor–like receptor—but not receptor activity modifying protein 1, VR1, and cannabinoid type 1 receptors—was increased in mesenteric resistance arteries in SHR compared with WKY (P<0.05). These data indicate that in addition to activation of cannabinoid type 1, anandamide may serve as an endogenous compound to stimulate VR1, leading to a decrease in blood pressure via CGRP release from sensory nerve terminals. Increased mesenteric CGRP receptor expression in SHR may account for increased sensitivity of blood pressure to anandamide and may serve as a compensatory response to buffer the increase in blood pressure in SHR. (Hypertension. 2003;41[part 2]:757-762.)

Key Words: anandamide ▪ receptors, vanilloid ▪ receptors, cannabinoid ▪ calcitonin gene-related peptide ▪ rats, spontaneously hypertensive

Anandamide was originally isolated from brain as an endogenous cannabinoid receptor ligand.1 Biosynthetic pathways for anandamide are also present outside the central nervous system, for example, in vascular endothelium and macrophages.2,3 Because of its therapeutic potential, there is a growing interest in the cardiovascular actions of endogenous cannabinoids. It has been demonstrated that anandamide elicits vasodilation of isolated small arteries2 and induces hypotension in anesthetized or conscious rats via activation of cannabinoid type 1 (CB1) receptor.4 However, numerous studies indicate that the cardiovascular effect of anandamide is not solely mediated by the CB1 receptor. For example, it has been shown that the vasodilator actions of anandamide in the mesenteric arterial bed are independent of the CB1 receptor.5 The potent CB1 agonist WIN55212-2 causes vasodilatation in the cat cerebral artery.6 WIN55212-2 does not cause vasodilatation in the rat mesenteric arterial bed, whereas the endogenous ligand anandamide does cause vasodilatation in this same location.7 Moreover, in CB1 knock-out mice, as well as CB1/CB2 double-knockout mice, anandamide-induced mesenteric vasodilatation is intact.8 Taken together, these data indicate that receptors distinctive from CB1 and CB2 receptors may mediate the vasodilatation and hypotensive effect of anandamide.

One of the potential candidates is the vanilloid receptor 1 (VR1), a ligand-gated ion channel that integrates multiple stimuli, including capsaicin, proton, and heat.9–11 This receptor is expressed almost exclusively in primary sensory nerves. It has been demonstrated that VR1 mediates cardiovascular effects of capsaicin, anandamide, and other vanilloid compounds, including vasodilatation in a variety of vascular beds.12,13 Activation of the VR1 receptor in primary sensory nerves by anandamide induces release of calcitonin gene-related peptide (CGRP) from sensory nerve endings and causes vasodilatation.13 These studies indicate that anandamide-induced activation of the VR1 receptor may play a significant role in blood pressure regulation.

In spontaneously hypertensive rats (SHR), the pathogenesis of hypertension appears to be heterogeneous, including the central nervous system, neurohumoral, and renal abnormali-
ties. It is known that a defect in sensory nerve function exists in SHR, which may constitute one of these abnormalities that contribute to the increase in blood pressure. It is unknown, however, whether altered sensory nerve function contributes to anandamide-induced changes in blood pressure in SHR. The present study was designed to answer two questions: (1) is anandamide-induced hypotension mediated, at least in part, by activation of the VR1 receptor in SHR; and (2) if so, what mechanism is underlying VR1-mediated hypotension induced by anandamide in SHR.

Methods

Animal Groups and Experimental Protocols

Eight- to 10-week-old male Wistar Kyoto rats (WKY) or SHR (Charles River Laboratories Inc, Wilmington, Mass) were allowed to acclimate for at least 3 days before experiment. The rats were anesthetized with ketamine and xylazine (80 and 4 mg/kg IP, respectively) before surgery of cannulation or urethane (1.5 g/kg IP) anesthesia with urethane to eliminate pain perception caused by capsaicin. MAP was recorded for 30 minutes, capsaicin or CAPZ were given by intravenous bolus injection under anesthesia with urethane to eliminate pain perception caused by capsaicin. MAP was recorded for another 1 hour after injections.

Experiment 1

WKY and SHR were divided into 2 groups for injection of capsaicin (100 µg/kg) alone or in combination with the specific VR1 receptor antagonist capsazepine (CAPZ, 1.5 mg/kg). After baseline MAP recording for 30 minutes, capsaicin or CAPZ were given by intravenous bolus injection under anesthesia with urethane to eliminate pain perception caused by capsaicin. MAP was recorded for another 1 hour after injections.

Experiment 2

WKY and SHR were divided into 3 groups for intravenous administration of methanandamide (MethA, 5 mg/kg) alone or in combination with either CAPZ (1.5 mg/kg) or the specific CB1 receptor antagonist SR141716A (1.5 mg/kg). Three hours after surgery, baseline MAP and its response to the above-mentioned drugs were determined with the rats fully awake and unrestrained.

Experiment 3

WKY and SHR were divided into 2 groups for administration of vehicle or MethA (5 mg/kg). Vehicle or MethA was given 3 hours after surgery with rats fully awake and unrestrained. Six to 7 minutes after the injection, when the prolonged depressor phase approached the lowest point, the rats were decapitated for collection of blood for plasma CGRP assay.

Experiment 4

WKY and SHR were decapitated without surgery for collection of dorsal root ganglia (DRG) for CGRP assay and mesenteric resistance arteries for Western blot analyses of VR1, CB1, calcitonin receptor–like receptor (CRLR), and receptor activity modifying protein 1 (RAMP1).

Radioimmunoassay

Blood (3 cc) was collected in EDTA tubes for plasma CGRP assay, and the cervical, thoracic, and lumbar DRG from each animal were immediately dissected and frozen in liquid nitrogen for determination of CGRP levels. To determine immunoactive CGRP in plasma and DRG, a commercially available rabbit anti-rat CGRP radioimmunoassay kit (Peninsula Laboratories Inc) was used. This antibody has 100% cross-reactivity with rat α-CGRP and 79% cross-reactivity with rat β-CGRP. There is no cross-reactivity with rat amylin, calcitonin, somatostatin, or substance P. The assay was performed as recommended by the supplier, and the total protein content in DRG in each sample was determined by the Bradford method (Bio-Rad).

Western Blot Analysis

Membrane protein of mesenteric resistance arteries was extracted, separated on a 10% sodium dodecyl sulfate–polyacrylamide gel, and transferred to polyvinylidene fluoride membrane, as in the method we previously described. Membranes were blocked overnight at 4°C in 5% milk washing solution (50 mmol/L Tris–HCl, 100 mmol/L NaCl, and 0.1% Tween-20 at pH 8.0) and then incubated with goat anti-rat VR1 polyclonal IgG (1:800, Santa Cruz Biotechnology), goat anti-human CB1 polyclonal IgG (1:500, Santa Cruz Biotechnology), rabbit anti-rat CRLR antiserum (1:5000, Alpha Diagnostic International), or rabbit anti-human RAMP 1 antiserum (1:5000, Alpha Diagnostic International) in blocking solution at 4°C overnight. After being washed extensively, the membranes were incubated with bovine anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology) or goat anti-rabbit IgG-HRP (1:5000, Santa Cruz Biotechnology) in blocking solution for 1 hour at room temperature. After being washed extensively, the membranes were incubated with ECL Plus (Amersham) and exposed to film. The films were developed and scanned by a Scanjet 5370C (Hewlett-Packard). The intensity of the reaction was determined by using the Image Quantity Program (Scion), and the final intensity was normalized by total protein loaded on membrane that was detected by Coomassie blue staining.

Immunohistochemistry

Mesenteric resistance arteries were cleaned of fat and connective tissues; were fixed with Zamboni’s fixative solution containing 2% formaldehyde, 15% picric acid, and 0.1 mol/L phosphate buffer (pH 7.4, 0.1 mol/L NaH2PO4 and 0.1 mol/L Na2HPO4) on ice for 2 hours; and were successively washed with dimethyl sulfoxide and PBS (three times for 10 minutes, respectively) at room temperature. The vessels were incubated in 0.4% Triton-X 100 (Sigma) and 5% fetal bovine serum (GIBCO BRL) in PBS for 30 minutes at room temperature to block nonspecific binding and then were incubated with rabbit anti-rat CGRP antiserum (1:400, Sigma) in PBS with 5% fetal bovine serum overnight at 4°C. After being washed with PBS, the vessels were incubated with anti-rabbit IgG conjugated with CY3 (1:300, Jackson Immuno Research) for 1 hour at room temperature. After being washed with PBS, the vessels were mounted to slides, and optical sections of the vessels were obtained by using a Zeiss Pascal confocal laser scanning microscope (Carl Zeiss Inc). The CY3 was excited by using a 543-nm HeNe laser (long-pass 560-nm emission filter). Pictures were restored in Adobe Photoshop (version 6.0, Adobe Systems).

Control experiments to verify the specificity of primary antibodies were performed as described previously. Preincubation of CGRP antiserum with its blocking peptides in 30-fold excess eliminated specific immunoreactivity. Omission of primary antibodies resulted in an absence of specific immunoreactivity.

Drugs

Methanandamide (Sigma) in ethanol was evaporated with nitrogen and then dissolved in ethanol (10% v/v), Tween-80 (10% v/v), and saline to be used fresh. SR141716A (provided by Dr Norbert E. Kaminski, Michigan State University, East Lansing, Mich) and capsaicin (Sigma) were dissolved in ethanol (10% v/v), Tween-80 (10% v/v), and saline. Capsazepine (Calbiochem) was dissolved in dimethyl sulfoxide (10% v/v), Tween-80 (10% v/v), and saline to make a stock solution of 15 mg/mL and was diluted in saline for intravenous injection.

Statistical Analysis

Values are expressed as mean±SE. Differences between groups were determined by t test or ANOVA followed by the Tukey–Kramer multiple comparison tests and were considered statistically significant at P<0.05.
Results

Baseline MAP was significantly higher in SHR (144±4 mm Hg) compared with WKY (108±3; n=8 to 10, *P<0.05) at the time of the experiments. To examine the effect of VR1 activation and inhibition by its specific agonist capsaicin and antagonist CAPZ on MAP in both SHR and WKY, MAP responses to intravenous administration of these 2 compounds were examined. Activation of the VR1 receptor by capsaicin caused a triphasic MAP response: transient hypotension followed by a brief pressor and more prolonged depressor phase. The prolonged depressor effect of capsaicin was significantly greater in SHR than in WKY (Figure 1, upper panel). CAPZ blocked the depressor effect of capsaicin by ~60% in SHR but had no effect in WKY (Figure 1, top).

Intravenous administration of MethA caused a similar triphasic MAP response as that caused by capsaicin in SHY and WKY. Also, the prolonged depressor effect of MethA was significantly greater in SHR than in WKY (Figure 1, center). Blockade of the VR1 receptor with CAPZ attenuated the depressor effect of MethA by ~50% in SHR but had no effect on WKY (Figure 1, center). Similarly, blockade of the CB1 receptor with SR141716A inhibited the depressor effect of MethA by ~60% in SHR but had no effect on WKY (Figure 1, bottom).

To determine whether MethA induces CGRP release in both SHR and WKY, plasma CGRP levels and CGRP contents in DRG were examined by radioimmunoassay. Although baseline CGRP content in DRG was not significantly different between SHR and WKY (Figure 2), MethA significantly increased plasma CGRP levels in both SHR and WKY, with no difference between the 2 strains (Figure 2). Immunohistochemistry staining revealed that CGRP-positive sensory nerves innervated the mesenteric resistance arteries in both SHR and WKY, and that the density of these nerve fibers appeared less in SHR (Figure 3).

To determine whether there is a difference in CGRP receptor protein expression in mesenteric resistance arteries between SHR and WKY, Western blot analysis was performed. One of the components of the CGRP receptor, CRLR, was significantly upregulated in SHR compared with WKY, whereas there was no difference in the other component, RAMP1, between SHR and WKY (Figure 4). Furthermore, Western blot revealed that VR1 and CB1 receptor protein expression in mesenteric resistance arteries was not significantly different between SHR and WKY (Figure 5).

Discussion

The goal of the present study was to determine whether anandamide-induced hypotension is mediated, at least in part,
by activation of the VR1 receptor in SHR, and if so, what mechanism is underlying VR1-mediated hypotension induced by anandamide in SHR. We found that (1) a specific VR1 receptor antagonist significantly blocks MethA-induced hypotension in SHR but not in WKY; (2) MethA induces CGRP release from sensory nerve endings in both SHR and WKY, but this effect of MethA is not distinctive between SHR and WKY; and (3) expression of CRLR is significantly upregulated in mesenteric resistance arteries in SHR compared with WKY, but there is no difference in mesenteric RAMP1, VR1, and CB1 receptor expression between SHR and WKY. Taken together, these data indicate for the first time that in addition to activation of the CB1 receptor, anandamide may serve as an endogenous compound to stimulate the VR1 receptor in sensory nerves, leading to a decrease in blood pressure via CGRP release from sensory nerve terminals. Increased CGRP receptor expression in mesenteric resistance arteries in SHR may account for increased sensitivity of blood pressure to anandamide and may serve as a compensatory response to buffer the increase in blood pressure in SHR.

Activation of the VR1 receptor with its specific agonist capsaicin leads to a remarkable decrease in blood pressures (~30 mm Hg) in SHR, whereas a much less dramatic decrease (~5 mm Hg) in blood pressure is observed in WKY. This potent hypotensive effect of capsaicin in SHR is specific in light of the fact that it can be blocked by the specific VR1 receptor antagonist CAPZ. These results indicate that activation of the VR1 receptor may serve as an effective means for lowering blood pressure in SHR. However, capsaicin, a derivative of red peppers, may not fulfill a role as an endogenous compound to activate the VR1 receptor. Therefore, we examined the role of anandamide, a known endogenous ligand for the CB1 receptor, in activating the VR1 receptor in sensory nerves in SHR and WKY.

Our data show that MethA, a stable analog of anandamide, indeed activates the VR1 receptor to lower blood pressure in SHR. This conclusion is based on the fact that (1) MethA induces a triphasic blood pressure response identical to that induced by capsaicin-activation of the VR1 receptor, and (2) blockade of the VR1 receptor with CAPZ impedes MethA-induced hypotension. These results are consistent with the hypothesis that anandamide, in addition to activating the CB1 receptor, leading to hypotension, may serve as an endogenous VR1 stimulator that plays a counter-regulatory role in attenuating the elevation in blood pressure in SHR. A subsequent question will be whether changes in blood pressure induced by MethA stimulation of VR1 are superimposed by that induced by MethA activation of CB1. Complete prevention of hypotensive effect of MethA by co-blockade of

Figure 3. Confocal imaging of perivascular sensory nerves stained positively with CGRP in mesenteric resistance arteries of SHR and WKY. The density of CGRP-positive perivascular sensory nerves (red) appeared to be lower in SHR than in WKY.

Figure 4. Western blot analysis of the calcitonin CRLR and RAMP1 in mesenteric resistance arteries in SHR and WKY. Values are mean±SE, n=7, P<0.05 vs WKY.

Figure 5. Western blot analysis of VR1 and CB1 in mesenteric resistance arteries in SHR and WKY. Values are mean±SE, n=4 to 5.
VR1 and CB1 would suggest a nonoverlapping mechanism. Otherwise, it would indicate that other pathways exist or that a more effective means of pharmacological blockade of VR1 and CB1 is required. Identification of these possibilities awaits for future studies that will apply both VR1 and CB1 receptor antagonists simultaneously in SHR and WKY.

Although VR1 and CB1 receptor expression in mesenteric resistance arteries is not altered in SHR compared with WKY, one possible hypotensive mechanism of MethA could be that activation of the VR1 receptor in sensory nerves leads to enhanced CGRP release from sensory nerve endings. CGRP, a potent vasodilator neuropeptide, participates in the regulation of vascular tone and regional organ blood flow, both under normal physiological conditions and in the pathophysiology of hypertension.23–24 A dense perivascular CGRP neural network is seen around the blood vessels in virtually every vascular bed.23–24 It is suggested that the vasodilator activity of CGRP is mediated by the release of this peptide from capsaicin-sensitive perivascular sensory nerve terminals.25 Furthermore, it has been demonstrated in vitro that activation of the VR1 receptor by anandamide causes vasodilation via stimulating CGRP release from capsaicin-sensitive sensory nerves, and that the CGRP receptor antagonist GRP
c
 inhibits anandamide-induced vasodilation.13 Although MethA also induces CGRP release in the current study, it may not account for increased blood pressure response to MethA in SHR, given that there is no difference in plasma CGRP levels after MethA administration between SHR and WKY. This is not surprising considering that CGRP levels in DRG and perivascular sensory nerves innervating mesenteric resistance arteries are not higher in SHR than in WKY.

To determine possible involvement of the postjunctional compartment, expression of the CGRP receptors in mesenteric resistance arteries was examined. We found that mesenteric CRLR, but not RAMP1, levels are significantly increased in SHR compared with WKY, supporting the hypothesis that enhanced hypotensive response to anandamide in SHR results from increased expression of the CGRP receptors in the target tissues. Indeed, it has been shown that the enhanced depressor response to CGRP occurs in SHR compared with WKY.26 Furthermore, it is known that CGRP activity is mediated by CRLR, which shares 55% homology with the calcitonin receptor. Although functional CGRP and adrenomedullin receptors are both derived from CRLR, the phenotype is determined by co-expression with a particular RAMP.27 A functional CGRP receptor is a CRLR co-expressed with RAMP1, whereas CRLR co-expressed with RAMP2 or RAMP3 produces an adrenomedullin receptor.27 Although RAMP1 expression in mesenteric resistance arteries is not different between SHR and WKY, co-expression of RAMP1 will constitute a functional CGRP receptor that is more sensitive to CGRP stimulation owing to upregulated CRLR component in SHR.

In conclusion, we have shown that blockade of the VR1 receptor hinders anandamide induced-hypotension in SHR, and that expression of the CGRP receptor is upregulated in mesenteric resistance arteries in SHR compared with WKY. These data indicate that anandamide may serve as an endogenous compound to stimulate the VR1 receptor, leading to a decrease in blood pressure via CGRP release from sensory nerve terminals. Increased CGRP receptor expression in mesenteric resistance arteries in SHR may account for increased sensitivity of blood pressure to anandamide and may serve as a compensatory response to buffer the increase in blood pressure in SHR.

Perspectives

The fact that the specific agonist of the VR1 receptor, capsaicin, possesses a remarkable hypotensive effect in SHR indicates that activation of the VR1 receptor may serve as an effective means for preventing the development of hypertension via increased release of sensory neurotransmitters. It follows that abnormalities in VR1 receptor expression, VR1 receptor–mediated signaling pathways, VR1 receptor–induced release of sensory neurotransmitters, or sensory neurotransmitter-receptor content and/or postreceptor signaling pathways would impact blood pressure regulation. Furthermore, given that anandamide may serve as an endogenous compound for activation of the VR1 receptor, it is conceivable that changes in circulating or tissue anandamide concentrations under a variety of pathophysiological conditions may alter VR1 function and therefore blood pressure. As a result, the search for endogenous VR1 activators or inhibitors and the study of regulation of these factors may hold a great promise for delineating molecular pathways underlying variation in blood pressure, as well as for a broad implication in vanilloid therapy that may be of value in treatment of hypertension, inflammatory hyperalgesia, and pain.

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