Anandamide Decreases Glomerular Filtration Rate through Predominant Vasodilation of Efferent Arterioles in Rat Kidneys

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Abstract. For determining the effects of anandamide (ANA) on renal hemodynamics and microcirculation, a clearance study was performed in Sprague-Dawley rats that received injections of ANA in doses of 15, 150, and 1500 pmol/kg. At doses up to 150 pmol/kg, ANA significantly decreased GFR and increased renal blood flow (RBF) without affecting mean arterial pressure (MAP). In the presence of the cannabinoid type 1 (CB1) receptor antagonist AM251, only the 15-pmol/kg dose significantly increased GFR and RBF without altering MAP, with higher doses having no effect on GFR, RBF, or MAP. By contrast, AM281, which antagonizes cannabinoid receptors nonselectively, inhibited the GFR, RBF, and MAP responses to ANA. The arteriolar responses to ANA were also assessed in vitro by the blood-perfused juxtamedullary nephron technique. Higher doses of ANA significantly increased the diameter of both afferent and efferent arterioles, whereas lower doses elicited predominant efferent arteriolar dilation. AM251 attenuated the afferent arteriolar response to ANA and inhibited the efferent arteriolar response to ANA, whereas AM281 inhibited the responses in both arterioles. The CB1 receptor mRNA was expressed in afferent arterioles, and immunohistochemical staining demonstrated the presence of CB1 receptors in both afferent and efferent arterioles. These results suggest that ANA causes afferent arteriolar dilation via both CB1 and non-CB1 receptors and greater efferent arteriolar dilation via CB1 receptors, resulting in a decreased GFR and an increased RBF without affecting MAP.

Septic shock is principally characterized by severe hemodynamic changes, including hypotension, decreased systemic vascular resistance, and compromised renal function. Although systemic hypotension seems to be involved in the decrease in GFR, some studies have demonstrated that decreased GFR during the early stage of sepsis is independent of systemic hemodynamics (1). Accordingly, some factors generated during sepsis may contribute to the compromised renal function independent of systemic hypotension.

Endogenous cannabinoid levels increase during lipopolysaccharide-induced septic shock and cause systemic hypotension through the cannabinoid type 1 (CB1) receptors (2). A recent study suggested that the polymyxin B–immobilized beads column used clinically to treat endotoxic shock may abolish the hypotensive, immunosuppressive, and cytotoxic effects during endotoxin shock by adsorbing anandamide (ANA), an endogenous cannabinoid generated by activated macrophages (3). Moreover, molecular studies have confirmed the intrarenal presence of an ANA signaling system that includes ANA, its putative precursor, the enzyme catalyzing the breakdown of ANA, and CB1 receptors (4–6). However, no studies of the direct effect of ANA on renal function have ever been reported.

The present study was designed to assess the direct effect of exogenous ANA on renal hemodynamics. Periglomerular microcirculatory responses to ANA were also assessed by using an in vitro blood-perfused juxtamedullary nephron technique, and CB1 receptors in the kidney were localized by immunohistochemical techniques and a reverse transcriptase–PCR (RT-PCR) method.

Materials and Methods

The present study was performed in accordance with the guidelines and practices established by the Keio University Animal Care and Use Committee. Normal male Sprague-Dawley rats (Charles River Japan, Kanagawa, Japan) that weighed 250 to 300 g were housed in wire cages and maintained on a 12:12-h light-dark cycle in a temperature-controlled room. Before the experiments, the rats had free access to water and standard rat diet (Oriental Yeast, Tokyo, Japan) that contains 110 µmol/g sodium.

Renal Clearance Study

Under anesthesia with pentobarbital sodium (50 mg/kg), tracheostomy and positive pressure ventilation were performed with an artificial respirator. The right jugular vein, right femoral artery, and right
carotid artery were respectively cannulated to infuse solutions, to monitor arterial pressure, and to inject bolus doses of ANA (Sigma, St. Louis, MO) and CB receptor antagonists. The bladder was catheterized via a suprapubic incision for urine collection. After surgery, the intravenous infusion was switched to isotonic saline solution containing 1% albumin, 1.5% p-aminohippurate sodium (PAH; Merck, Sharp & Dohme, West Point, PA), and 7.5% inulin (Inutest; Laevosan, Linz, Austria). A 60-min equilibration period was provided after the completion of surgery.

The experimental protocol consisted of three 30-min clearance periods to assess control renal function. Between the second and third periods, the rats received an intra-arterial injection of saline, the selective CB1 receptor antagonist AM251 (1500 pmol/kg; Tocris Cookson, Ballwin, MO), or the nonselective CB receptors antagonist AM281 (1500 pmol/kg; Tocris Cookson). The rats were then given three intra-arterial bolus injections of increasing doses of ANA (15, 150, and 1500 pmol/kg) at 40-min intervals. After a 10-min delay, a 30-min experimental clearance period was provided for each dose of ANA, and an arterial blood sample was collected at the midpoint of each 30-min clearance period to calculate inulin clearance (Cinulin) and PAH clearance (CPAH). The microhematocrit of all arterial blood samples was measured and remained constant throughout the experiment. Cinulin was used as an index of GFR, and CPAH was used as an index of renal blood flow (RBF) because of the constant hematocrit. All drugs were given in total volume of 100 μl.

Additional experiments were performed to investigate the role of renal sympathetic nerves in renal hemodynamic responses to ANA. Bilateral kidney denervation was performed, as described previously (7). The intrarenal norpinephrine content was confirmed to be significantly lower in the renal-denervated rats (20.0 ± 8.9 ng/g tissue; n = 5) than in the control rats (165.8 ± 13.5 ng/g tissue; n = 4).

**Measurement of Afferent and Efferent Arteriolar Diameter**

Afferent and efferent arteriolar diameters were measured in vitro by the blood-perfused juxtaglomerular nephron technique combined with videomicroscopy, as described previously (8). Afferent arteriolar diameters were measured at sites 90 to 150 μm upstream from the glomerulus, and efferent arteriolar diameters were measured at sites within 100 μm of the glomerulus, before the first branch. A 10-min equilibration period was allowed before initiating each experimental protocol. The average diameter during the final 2 min of each 5-min period was used to statistically analyze steady-state responses.

Afferent and efferent arteriolar diameters were measured before and during superfusion with increasing doses of ANA (0.1, 1, and 10 μM). For investigating the involvement of CB1 and CB2 receptors in ANA-mediated afferent and efferent arteriolar relaxation, the ANA responses were assessed again in the presence of 1 nM AM251 or 1 nM AM281.

**Immunohistochemical Localization of CB1 Receptors**

For detecting CB1 receptors, kidneys were perfused in situ with saline and fixed in Bouin’s solution. Sections were immersed in 3% H2O2 in methanol to inhibit endogenous peroxidase and then flooded with 5% BSA in PBS to inhibit nonspecific reactions. Polyclonal goat anti-CB1 receptor antibodies (sc-10066; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution) were used as the primary antibodies, and a biotinylated polyclonal donkey anti-goat antibody (1:500 dilution) was applied as the secondary antibody. Immunoreaction was performed using a Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA) and visualized with 3-amino-9-ethylcarbazole (DAKO, Carpinteria, CA) as a substrate, followed by light counterstaining with hematoxylin. We discriminate efferent arterioles from afferent arterioles on the basis of the criteria that the efferent arterioles have a significant intraglomerular segment that runs through the glomerular stalk, whereas the afferent arterioles divide into several primary capillary branches strictly at the entrance of the glomerulus.

**Detection of CB1 Receptor mRNA in Afferent Arterioles**

Afferent arterioles were prepared as described previously (9). In brief, the kidneys were cleared of blood by perfusion in situ with ice-cold low-calcium physiologic salt solution (PSS; pH 7.35) followed by an identical solution that contained 1% Evans blue. The renal cortex was sieved with a 180-μm nylon mesh, and the retentate was washed with ice-cold low-calcium PSS. The vascular tissue that remained on the sieve was transferred to the solution that contained 0.075% collagenase (Calbiochem, La Jolla, CA), 0.02% dithiothreitol (Sigma), 0.2% soybean trypsin inhibitor (type 1-S; Sigma), and 0.1% BSA dissolved in low-calcium PSS and incubated for 30 min at 37°C. The vascular tissue was transferred to a 70-μm nylon mesh and washed with ice-cold low-calcium PSS. The retained vascular tissue was transferred to a dish that contained ice-cold low-calcium PSS, and afferent arterioles were collected on the stage of a Nikon microscope (model SMZ800) and stored at −80°C. Frozen afferent arterioles were homogenized in Trizol reagent (Life Technologies, Grand Island, NY) and extracted following the manufacturer’s instructions. RNA was resuspended in DEPC-H2O and stored at −20°C until use. DNase-I–treated total RNA was subjected to RT-PCR using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA). Primer sequences used were as follows: forward primer: 5′-ATTTCAGCAAGGACACCCA-3′; reverse primer: 5′-CATTCGAAGCCACGTAAGGA-3′.

**Quantitative Analysis of CB1 Receptors in Afferent and Efferent Arterioles**

The right kidneys from a Sprague-Dawley rat was perfusion fixed and removed. It was further fixed in 3.7% paraformaldehyde for 15 min at room temperature, then vibratome-sectioned into 100-μm sections, followed by 0.2% Triton X100 (5 min) for permeabilization. For fluorescence staining, the sections were washed several times in PBS with 0.1%BSA (PBS-BSA) and incubated with primary antibodies (goat polyclonal CB1) in PBS-BSA for 1 h at room temperature followed by overnight at 4°C and washed again in PBS-BSA, and fluorescence-labeled secondary antibody (donkey anti-goat–Texas Red; Jackson Laboratory, Bar Harbor, ME) was applied, followed by 1 h of incubation at room temperature and washing. A control of only secondary antibody was done simultaneously. They were mounted in Antifade (Molecular Probes, Eugene, OR). Microscopy was performed on a BioRad two-photon microscope. The results were analyzed using Voxx software (10) and Metaphor (Fryer, Huntley, IL).

**Statistical Analyses**

Statistical comparisons within a group were made by one-way ANOVA for repeated measures followed by the Newman-Keuls post hoc test. Differences between two groups were evaluated by two-way ANOVA for repeated measures combined with the Newman-Keuls post hoc test. P < 0.05 was considered significant. Data are reported as means ± SEM.
Results

Effect of ANA on Arterial Pressure and Renal Hemodynamics

Figure 1 shows the changes in mean arterial pressure (MAP) during intra-arterial injection of increasing doses of ANA in the control rats (n = 14) and bilaterally renal denervated rats (n = 7). Before ANA administration, the MAP in the control rats and bilaterally renal denervated rats averaged 92 ± 2 and 93 ± 2 mmHg, respectively, and it was unaltered by ANA up to 150 pmol/kg. The 1500-pmol/kg dose, however, elicited a significant decrease in MAP in both groups, to 84 ± 3 and 73 ± 6 mmHg, respectively.

Figure 2 shows the responses of Cinulin and CPAH to intra-arterial injection of ANA in the control rats (n = 14) and bilaterally renal denervated rats (n = 7). In the control rats, the 15-, 150-, and 1500-pmol/kg doses of ANA significantly decreased Cinulin from 1.49 ± 0.28 to 0.98 ± 0.13, 0.76 ± 0.12, and 0.78 ± 0.20 ml/min per g kidney weight, respectively, and significantly increased CPAH from 6.1 ± 0.7 to 9.9 ± 1.6, 9.3 ± 1.8, and 7.4 ± 1.7 ml/min per g kidney weight, respectively. In the bilaterally renal denervated rats, the ANA injections also decreased Cinulin, from 1.26 ± 0.22 to 1.02 ± 0.25, 1.00 ± 0.24, and 0.59 ± 0.06 ml/min per g kidney weight, respectively, and increased CPAH, from 4.8 ± 1.0 to 8.6 ± 1.6, 9.6 ± 1.4, and 8.5 ± 1.3 ml/min per g kidney weight, respectively. Urinary sodium excretion (UNaV) was significantly higher in the bilaterally renal denervated rats (19.8 ± 2.6 mmol/d) than in the control rats (9.3 ± 1.3 mmol/d), but the ANA injections had no significant effect on UNaV in either group.

Renal Hemodynamic Responses to ANA during Inhibition of CB Receptors

Figure 3 shows the MAP responses to the ANA injections in rats that received an intra-arterially injection of AM251 (n = 13) or AM281 (n = 15) at the 1500-pmol/kg dose. The MAP was unaffected by the ANA injections in either group.

Figure 4 shows the responses of Cinulin and CPAH to the intra-arterial ANA injections in the rats that were treated with

AM251 (n = 13) or AM281 (n = 15). In the presence of AM251, the 15-pmol/kg dose of ANA significantly increased Cinulin from 2.57 ± 0.54 to 8.35 ± 2.35 ml/min per g kidney weight and CPAH from 5.4 ± 1.4 to 8.9 ± 2.3 ml/min per g kidney weight, but the higher ANA doses (150 and 1500 pmol/kg) did not affect either Cinulin (4.59 ± 2.84 and 2.23 ± 0.82 ml/min per g kidney weight, respectively) or CPAH (8.0 ± 2.1 and 3.7 ± 0.3 ml/min per g kidney weight, respectively).
In the presence of AM281, neither Cinulin nor CPAH was influenced by any doses of ANA. Before and after the injections of 15, 150, and 1500 pmol/kg ANA, Cinulin averaged 2.10 ± 0.42, 2.28 ± 0.52, 1.60 ± 0.32, and 1.72 ± 0.39 ml/min per g kidney weight, respectively, and CPAH averaged 11.7 ± 3.7, 12.8 ± 3.6, 11.8 ± 3.8, and 10.4 ± 5.4 ml/min per g kidney weight, respectively. The ANA injections had no effect on UNaV in the presence of either AM251 or AM281.

Arteriolar Responses to ANA before and during CB1 Receptor Inhibition

Figure 5 shows the afferent (n = 9) and efferent (n = 6) arteriolar responses to graded concentrations of ANA of 0.1, 1, and 10 μM before and during superfusion with the CB1 receptor antagonist AM251 (1 nM). Basal afferent arteriolar diameters averaged 12.1 ± 1.2 μm and were unaffected by ANA at the 0.1- and 1-μM concentrations. Afferent arteriolar diameter during superfusion with 0.1 and 1 μM ANA averaged 12.8 ± 1.2 and 13.8 ± 1.3 μm, respectively, but the 10-μM concentration significantly increased afferent arteriolar diameters to 15.7 ± 1.2 μm. The selective CB1 receptor blockade with AM251 had no effect on afferent arteriolar diameter, but it significantly attenuated the dilator responses of afferent arterioles to 10 μM ANA. In the presence of AM251, superfusion with 10 μM ANA increased afferent arteriolar diameter from 12.3 ± 1.2 to 13.1 ± 1.2 μm, but the increase was significantly smaller than that observed before administration of AM251.

Basal efferent arteriolar diameter averaged 13.8 ± 1.2 μm, and in response to 0.1, 1, and 10 μM ANA, it significantly increased to 16.0 ± 1.1, 19.2 ± 1.0, and 20.9 ± 1.5 μm, respectively. At each concentration of ANA, the increase in efferent arteriolar diameter was significantly greater than the increase in afferent arteriolar diameter. AM251 did not alter efferent arteriolar diameter, and it completely inhibited the efferent arteriolar response to ANA. In the presence of AM251, efferent arteriolar diameter before and during superfusion with 0.1, 1, and 10 μM ANA averaged 12.9 ± 0.7, 13.0 ± 1.2, 12.8 ± 0.8, and 12.7 ± 1.2 μm, respectively.

Arteriolar Responses to ANA before and during CB1 and 2 Receptors Inhibition

Figure 6 shows afferent (n = 7) and efferent (n = 5) arteriolar responses to graded ANA concentrations of 0.1, 1, and 10 μM before and during superfusion with 1 nM AM281, which antagonizes CB receptors nonselectively. Basal afferent arteriolar diameter averaged 12.2 ± 1.1 μm and was unaffected by 0.1 μM ANA. Afferent arteriolar diameter during superfusion with 0.1 μM ANA averaged 11.9 ± 0.9 μm, but in response to 1 and 10 μM ANA, it significantly increased to 12.9 ± 0.9 and 15.3 ± 0.8 μm. The nonselective blockade of CB receptors with AM281 had no effect on afferent arteriolar...
diameter but completely inhibited the afferent arteriolar responses to ANA. In the presence of AM281, afferent arteriolar diameter before and during superfusion with 0.1, 1, and 10 μM ANA averaged 12.0 ± 1.0, 12.1 ± 0.8, 12.2 ± 1.0, and 12.1 ± 0.9 μm, respectively.

Baseline efferent arteriolar diameters averaged 12.0 ± 2.0 μm, and 0.1, 1, and 10 μM ANA significantly increased efferent arteriolar diameter to 13.4 ± 1.9, 16.0 ± 1.5, and 17.2 ± 1.3 μm, respectively. The dilator responses to all concentrations of ANA in efferent arterioles were significantly greater than in afferent arterioles. AM281 did not alter efferent arteriolar diameter, but it completely inhibited the efferent arteriolar responses to ANA. In the presence of AM281, efferent arteriolar diameter before and during superfusion with 0.1, 1, and 10 μM ANA averaged 12.0 ± 2.1, 12.5 ± 2.2, 12.2 ± 1.9, and 11.4 ± 1.7 μm, respectively.

Immunohistochemical Localization of CB1 Receptors

The CB1 receptors were found exclusively in the intrarenal vascular systems, and there was no staining in the glomeruli, tubules, or interstitium. Figure 7, A and B, shows the similar staining for CB1 receptors in both afferent and efferent arterioles. These stainings were not seen when the antibodies were preabsorbed with competing peptide for CB1 receptors (sc-10066P; Santa Cruz Biotechnology; data not shown). There was no staining of CB1 receptor in the glomeruli, tubules, or interstitium.

Detection of CB1 Receptor mRNA in Afferent Arterioles

The RT-PCR analysis verified the mRNA expression of CB1 receptor in afferent arterioles. Figure 7C shows the 169-bp band (arrow), which is close to the predicted RT-PCR product size for CB1 receptor, in the kidneys and brain. These bands disappeared in the absence of RT.

Quantitative Analysis of CB1 Receptors in Afferent and Efferent Arterioles

Figure 8 shows immunofluorescent staining of CB1 receptors in both afferent and efferent arterioles. Quantitative analysis using the two-photon microscopy (n = 4) revealed a slightly but significantly greater immunostaining for CB1 receptors in afferent arterioles (122.9 ± 3.9 densitometric units) than in efferent arterioles (100.4 ± 7.2 densitometric units).

Discussion

Increased ANA levels in the blood of sepsis patients cause systemic vasodilation that results in hypotensive shock. The results of the present study consistently demonstrated a significant decrease in MAP in response to administration of 1500 pmol/kg ANA. In addition, at doses of 150 pmol/kg and lower, ANA administration significantly increased CPAH and decreased Cinulin without affecting MAP or hematocrit, and similar results were observed in the rats that were subjected to bilateral renal denervation. These results suggest that ANA increases RBF and decreases GFR independent of its effects on BP and renal nerves and that intrarenal vasculature is more sensitive to ANA than systemic resistance arteries.

ANA significantly increased RBF, and the nonselective CB receptors antagonist AM281 inhibited the increases in RBF at all doses of ANA. The present in vitro studies demonstrated that ANA elicits significant vasodilation in afferent arterioles, which account for ~90% of the preglomerular vascular resistance. In addition, the ANA-induced afferent arteriolar dilation was attenuated by the blockade of CB1 receptors with AM251 and inhibited by the nonselective blockade of CB receptors with AM281. These results suggest that the RBF responses to ANA are wholly dependent on the CB receptors-mediated afferent arteriolar dilation.

Intra-arterial ANA injections caused a significant decrease in GFR, represented by Cinulin, but did not alter UNaV, suggesting that the renal tubular system may regulate urinary sodium excretion normally even in the presence of ANA. This concept was supported by our immunohistochemical studies that showed staining of CB1 receptors in the renal vasculature system alone with no staining in the glomeruli, tubules, or interstitium. It therefore is likely that ANA has no luminal effects and that the changes in GFR are due to the effect of ANA on the renal vasculature. Lower doses of ANA induced a vasodilation only in efferent arterioles, and higher doses of ANA increased afferent arteriolar diameter and dilated efferent arterioles to a greater extent. Because predominant afferent arterioles are wholly dependent on the CB receptors-mediated afferent arteriolar dilation.
arteriolar vasodilation decreases intraglomerular pressure, the differential effects of ANA on afferent and efferent arterioles seem to account for the decreased GFR.

Why does ANA dilate efferent arterioles so much more than afferent arterioles? To answer this question, we performed pharmacologic experiments and immunohistochemical staining of CB1 receptors and assessed the role of CB receptors in the mechanism by which ANA exerts different effects on afferent and efferent arterioles. The pharmacologic studies revealed that CB1 receptors contribute to the afferent and efferent arteriolar dilator responses to ANA, whereas non-CB1 receptors contribute to the dilator response to ANA in afferent arterioles alone. These findings were consistent with the results of immunohistochemical staining demonstrating that both afferent and efferent arterioles have CB1 receptor immunoreactivities. In addition, RT-PCR analysis showed the CB1 receptor mRNA expression in afferent arterioles, and the two-photon microscopy showed a greater immunostaining for CB1 receptors in afferent arterioles compared with efferent arterioles. In afferent arterioles, therefore, non-CB1 receptors may antagonize the function of CB1 receptors.

Recent studies demonstrated the absence of CB2 receptor in rat kidneys (11) and suggested the existence of a nonclassical CB receptor that is neither a CB1 receptor nor a CB2 receptor but is also blocked by an ordinary CB receptor antagonist (12). Because the CB receptor antagonist SR141716A also inhibits Ca$^{2+}$-activated K$^+$ channels and voltage-dependent K$^+$ channels (12), endothelium-derived hyperpolarizing factors may contribute to the function of the ANA-specific non-CB1, non-CB2 receptors. Because endothelium-derived hyperpolarizing factors predominantly modulate afferent, not efferent arterioles (13,14), it is possible that endothelium-derived hyperpolarizing factors mediate the function of non-CB1 receptors, which is blocked by AM281.

The great increase in Cinulin after 15 pmol/kg ANA was observed in the presence of the CB1 receptor antagonist AM251 and disappeared in the presence of the nonselective CB receptor antagonist AM281. These results suggest that low...
doses of ANA may increase GFR through the non-CB1 receptor–mediated afferent arteriolar dilation. However, higher doses of ANA did not affect Cinulin in the presence of AM251. Excessive activation of afferent arteriolar non-CB1 receptors may cause excessive hypoperfusion in the kidneys, and the alterations of GFR and RBF may resume as a result. Further studies will be needed to clarify the function of non-CB receptors in the kidneys.

In conclusion, the present study demonstrated for the first time that ANA significantly decreases GFR and increases RBF independent of its effects on BP and renal nerves and that ANA elicits vasodilatation in afferent and efferent arterioles through CB receptors with a greater effect on efferent arterioles. Because the CB receptors were present exclusively in the renal vascular system, the ANA-induced predominant vasodilatation in efferent arterioles accounts for the decrease in GFR.

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