1521-0103/349/1/29–38$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.113.209213
J Pharmacol Exp Ther 349:29–38, April 2014

The Novel Endocannabinoid Receptor GPR18 Is Expressed in the Rostral Ventrolateral Medulla and Exerts Tonic Restraining Influence on Blood Pressure[S]

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Received August 30, 2013; accepted January 15, 2014

ABSTRACT

Systemic administration of the G-protein–coupled receptor 18 (GPR18) agonist abnormal cannabidiol (Abn CBD) lowers blood pressure (BP). Whether GPR18 is expressed in the central nervous system (CNS) and plays a role in BP control is not known despite the abundance of the GPR18 ligand *N*-arachidonoyl glycine (NAGly) in the CNS. Therefore, we first determined whether GPR18 is expressed in the presympathetic tyrosine hydroxylase (TH) immunoreactive (ir) neurons of the brainstem cardiovascular regulatory nuclei. Second, we investigated the impact of GPR18 activation and blockade on BP and heart rate (HR) and neurochemical modulators of sympathetic activity and BP. Immunofluorescence findings revealed GPR18 expression in TH-ir neurons in the rostral ventrolateral medulla (RVLM). Intra-RVLM GPR18 activation (Abn CBD) and blockade (O-1918, 1,3-dimethoxy-5-methyl-2-[(1*R*,6*R*)-3-methyl-6-(1-methylethenyl)-2-,cyclohexen-1-yl] benzene) elicited dose-dependent reductions and elevations in BP, respectively, along with respective increases and decreases in HR in conscious male Sprague-Dawley rats. RVLM GPR18 activation increased neuronal adiponectin (ADN) and NO and reduced reactive oxygen species (ROS) levels, and GPR18 blockade reduced neuronal ADN and increased oxidative stress (i.e., ROS) in the RVLM. Finally, we hypothesized that the negligible hypotensive effect caused by the endogenous GPR18 ligand NAGly could be due to concurrent activation of CB_1R in the RVLM. Our findings support this hypothesis because NAGly-evoked hypotension was doubled after RVLM CB_1R blockade (SR141716, rimonabant). These findings are the first to demonstrate GPR18 expression in the RVLM and to suggest a sympathoinhibitory role for this receptor. The findings yield new insight into the role of a novel cannabinoid receptor (GPR18) in central BP control.

Introduction

Endogenous and exogenous cannabinoids exert complex cardiovascular effects that are due, at least partly, to their activation of diverse cannabinoid receptors (CBRs) and their location within the cardiovascular system and different brain nuclei (Randall et al., 2004). The two classic CBRs (CB_1R and CB_2R) are implicated in behavior and cardiovascular regulation (Randall et al., 2002). However, the cardiovascular responses might be confounded by the use of anesthetics because CBs produce hypotension in anesthetized animals (Varga et al., 1995), which is consistent with vasorelaxation in vitro (Jarai et al., 1999), but produce pressor response in conscious rats (Gardiner et al., 2002; Ibrahim and Abdel-Rahman, 2011). Further, many studies implicated several novel receptors in the diverse cardiovascular effects of synthetic and natural cannabinoids (Offertáler et al., 2003). For example, Abn CBD caused mesenteric vasodilation and hypotension in mice lacking CB_1/CB_2 receptors via the activation of a novel CB receptor (Jarai et al., 1999). This new G_i/G_o-coupled receptor, which mediates endothelium-dependent vasodilation (Begg et al., 2005; Mackie and Stella, 2006), has been named the endothelial "anandamide receptor," or the "Abn CBD" receptor. Recent findings indicate that *N*-arachidonoyl glycine (NAGly), Abn CBD, and Δ^9-tetrahydrocannabinol (THC) (McHugh et al., 2012) act as selective agonists, whereas O-1918, an analog of cannabidiol (CBD), acts as an antagonist at this receptor (Offertáler et al., 2003; McHugh et al., 2010).

Recent studies have identified the orphan G-protein–coupled receptor GPR18 as the Abn CBD receptor and NAGly as its endogenous ligand (Kohno et al., 2006; McHugh et al., 2010; McHugh, 2012). It is possible that Abn CBD and O-1918 might act via another putative cannabinoid receptor, GPR55 (Johns

This research was supported by the Department of Pharmacology and Toxicology at the Brody School of Medicine, East Carolina University; and the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant 2R01-AA07839-19].
dx.doi.org/10.1124/jpet.113.209213.
[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: Abn CBD, abnormal cannabidiol (*trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol); ADN, adiponectin; AEA, *N*-arachidonoylethanolamine; BP, blood pressure; CBD, cannabidiol; CB_1R, cannabinoid receptor 1; CB_2R, cannabinoid receptor 2; CNS, central nervous system; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DHE, dihydroethidium; DMSO, dimethylsulfoxide; GPCR, G-protein–coupled receptor; GPR18, G-protein–coupled receptor 18; HR, heart rate; ir, immunoreactive; MAP, mean arterial pressure; NAGly, *N*-arachidonoyl glycine; NO, nitric oxide; O-1918, 1,3-dimethoxy-5-methyl-2-[(1*R*,6R)-3-methyl-6-(1-methylethenyl)-2-,cyclohexen-1-yl]benzene; RVLM, rostral ventrolateral medulla; SR141716, rimonabant; TH, tyrosine hydroxylase.

et al., 2007; Godlewski et al., 2009). However, more recent studies showed that although GPR18 and GPR55 share some ligands, they do not respond to them in the same manner or with the same efficacy (McHugh et al., 2010; Okuno and Yokomizo, 2011).

GPR18 is found in humans, rodents, and canines (Gantz et al., 1997), and its mRNA is most abundantly expressed in the testis, spleen, and brainstem, among other tissues (Vassilatis et al., 2003). Although GPR18 mRNA is expressed in human and rodent brainstem (Vassilatis et al., 2003) and NAGly is abundant in the brain (Huang et al., 2001), no studies have been reported on the expression and function of GPR18 in brainstem and cardiovascular/sympathetic activity regulating nuclei. Notably, NAGly or Abn CBD activation of GPR18, which is coupled to the $G_i/_o$ family (Kohno et al., 2006), enhances ERK1/2 phosphorylation and PI3K/Akt signaling (Offertáler et al., 2003; Mo et al., 2004), as well as glycinergic transmission in the nervous system (Jeong et al., 2010). Further, NAGly causes vasorelaxation via NO release in rat small mesenteric arteries (Parmar and Ho, 2010), and *N*-palmitoyl glycine (a palmitic acid conjugate of NAGly) increases the levels of NO (Rimmerman et al., 2008). Whether these signaling effects result in functional changes in sympathetic activity and ultimately in BP has not been investigated. Notably, the findings that the endogenous GPR18 ligand NAGly has the potential to increase anandamide (AEA) (endogenous CB_1R agonist) (Burstein et al., 2002) and that central CB_1R activation increases BP (Ibrahim and Abdel-Rahman, 2012) might influence the final BP response mediated by NAGly activation of RVLM GPR18.

The main objectives of the present study were to determine whether GPR18 is expressed in RVLM presympathetic neurons and to elucidate its role in central control of BP. To achieve these goals, we conducted integrative dose-response studies that permitted measurements of BP and HR responses caused by direct activation and/or blockade of the RVLM GPR18 in conscious rats. We also investigated the possibility that concurrent activation of RVLM CB_1R, which mediates sympathoexcitation, might explain the dampened hypotensive response produced by the endogenous GPR18 ligand NAGly. Finally, the integrative pharmacologic studies were complemented with ex vivo neurochemical studies to elucidate the molecular mechanisms implicated in the central GPR18-mediated hypotensive response.

Materials and Methods

Preparation of the Rats. Male Sprague-Dawley rats (300–350 g; Charles River Laboratories, Raleigh, NC) were used in the present study. All rats were housed two per cage in a room with a controlled environment at a constant temperature of 23 ± 1°C, humidity of 50% ± 10%, and a 12-hour light/dark cycle. Food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical, postoperative care, and experimental procedures were performed in accordance with, and approved by, the Institutional Animal Care and Use Committee and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 2011). Arterial catheterization, intra-RVLM cannulation and BP measurements were performed as reported in our previous studies (Mao and Abdel-Rahman, 1995; Zhang and Abdel-Rahman, 2002) and as detailed in the Supplemental Material.

Western Blot and Neurochemical Studies. Animals received a lethal dose of sodium pentobarbital (intraperitoneally), and after decapitation, brains were removed, flash-frozen in 2-methylbutane on dry ice, and stored at −80°C until use as detailed in the Supplemental Material.

Immunohistochemistry. The procedure reported in Current Protocols in Neuroscience for immunohistochemistry for light microscopy (Ince et al., 1997) was followed and detailed in the Supplemental Material.

Immunofluorescence. Colocalization studies were conducted according to the protocol used in previous reports (Wang and Abdel-Rahman, 2005; Matias et al., 2008) and as detailed in the Supplemental Material.

Measurement of Nitrate/Nitrite. RVLM punches were obtained from the rats of different experimental groups and homogenized in 300 μl of phosphate-buffered saline. The homogenate was centrifuged (14,000 rpm) for 20 minutes, and the protein in the supernatant was quantified using a Bio-Rad protein assay system. The supernatant (140 μl) was ultra-filtered using Amicon Centrifugal Filter Units (10 kDa) and centrifuged (14,000 rpm) for 1 hour. The NOx content was measured using a nitrate colorimetric assay kit according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI) and as detailed in reported studies (Misko et al., 1993; El-Mas et al., 2009).

Dihydroethidium Staining for ROS Detection. For measurement of ROS, fresh unfixed brainstem sections (20 μm) were incubated with 10 μM dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO_2 in a moist chamber for 30 minutes. Positive and negative controls were used to validate the assay (see "Experimental Groups and Protocols"). Images were visualized with a Zeiss LSM 510 microscope. Three to five images were acquired from four brainstem sections for each experimental condition. Quantification was conducted using ImageJ Software (National Institutes of Health) and changes in total fluorescence intensity, normalized to control, were calculated as reported (Collin et al., 2007).

Measurement of Reactive Oxygen Species by DCFH-DA. RVLM specimens from treated and control groups were homogenized in phosphate-buffered saline. The homogenate was centrifuged (14,000 rpm) for 20 minutes. Protein in the supernatant was quantified using a Bio-Rad protein assay system. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) was dissolved in dimethylsulfoxide (DMSO) (12.5 mM) and kept at −80°C in the dark. It was freshly diluted with 50 mM phosphate buffer (pH 7.4) to 125 μM before experiment. DCFH-DA was added to RVLM homogenate supernatant (10 μl) in a 96-well microtiter plate for a final concentration (25 μM). 2′,7′-Dichlorofluorescein (DCF) was used for a six-point standard curve. Quantification was conducted by examining fluorescence intensity using a microplate fluorescence reader at excitation 485 nm/emission 530 nm. Kinetic readings were recorded for 30 minutes at 37°C. ROS level was calculated by relative DCF fluorescence per microgram of protein. Positive and negative controls were used to validate the assay as in our previous studies (McGee and Abdel-Rahman, 2012).

Experimental Groups and Protocol

Anatomic Expression of GPR18 in the RVLM. Coronal sections were obtained from naïve rats ($n = 5$) for detecting GPR18 protein by immunohistochemistry and by Western blotting as described under *Materials and Methods*. Positive (testis and spleen) and negative (liver) controls, based on reported studies (Gantz et al., 1997), were simultaneously run with RVLM GPR18 to confirm the Western blot findings. Furthermore, we used the GPR18 blocking peptide, recommended by the manufacturer, to verify the GPR18 antibody specificity. Spatial distribution of GPR18, in relation to tyrosine hydroxylase (TH)-ir neurons in the RVLM was investigated by dual labeling immunofluorescence in brainstem sections containing the RVLM as described under *Materials and Methods* and in our previous studies to verify the colocalization of the c-Fos immunoreactive cell nucleus and TH-ir neurons in the RVLM (Ibrahim and Abdel-Rahman, 2011).

Functional Role of RVLM GPR18 in BP and HR Regulation. No studies on the effect of the activation or blockade of RVLM GPR18 on blood pressure in conscious or even anesthetized rats have been reported. Therefore, we conducted preliminary studies in conscious instrumented rats to identify a dose range for the microinjected GPR18 agonist Abn CBD on BP. Thereafter, four groups of conscious unrestrained rats (n = 5 to 6) were used for investigating the dose-BP/HR responses elicited by intra-RVLM microinjections of the GPR18 agonists NAGly (0.5, 1, 2, or 4 μg) or Abn CBD (0.2, 0.4, or 0.8 μg) or the antagonist O-1918 (0.2, 0.4, 0.8 μg); control rats received equal volume (80 nl) of vehicle (methyl acetate). After stabilization of BP and HR at baseline, the rats in a particular group received intra-RVLM microinjections of only the GPR18 agonist or antagonist, and control rats received equal amount of the vehicle. Three additional groups of rats (n = 6 each) were included to determine the involvement of baroreflexes in the tachycardic and bradycardic response observed after Abn CBD and O-1918 microinjection, respectively. Vagal (1 mg/kg i.v. atropine) and β-adrenergic receptor blockade (1 mg/kg i.v. propranolol), which abolishes baroreflex mediated bradycardia and tachycardia, according to established protocol (Coleman, 1980), was induced in all rats. Thirty minutes after atropine and propranolol administration, the rats in a particular group received intra-RVLM Abn CBD (0.2, 0.4, or 0.8 μg), O-1918 (0.2, 0.4, or 0.8 μg) or equal volume of vehicle.

Effect of O-1918 on the BP and Neurochemical Responses Elicited by Intra-RVLM Abn CBD. Based on the dose-response findings of experiment 2, as already discussed, 0.4 μg of Abn CBD or O-1918 (80 nl) was used in this experiment to test the hypothesis that enhancement of adiponectin and NO generation in the RVLM underlie the GPR18-mediated hypotensive response. The effects of RVLM GPR18 activation (Abn CBD) on BP and neurochemical responses were investigated in the absence or presence of the selective GPR18 blockade (O-1918) in four groups of conscious male rats (n = 5 to 6 each). After stabilization of BP and HR at baseline, the rats in a particular group received intra-RVLM vehicle or Abn CBD (0.4 μg) 30 minutes after methyl acetate (vehicle) or O-1918 (0.4 μg). BP and HR were monitored after Abn CBD administration, and the animals were euthanized during the hypotensive response in the Abn CBD group and the corresponding time in the O-1918 + Abn CBD group. The brains were collected and processed for neurochemical studies.

Effect of Microinjecting ADN into the RVLM on BP, NO, and ROS Levels. In this experiment, we investigated the impact of microinjecting ADN into the RVLM on mean arterial pressure and heart rate. Animals in this experiment received increasing doses of adiponectin (0.25, 0.5, 1, 2, and 4 pmol); the doses were based on adding two lower and two higher doses than the reported 1 pmol of ADN microinjected into the area postrema (Fry et al., 2006). Neurochemical effects of ADN were investigated in the brains collected after the conclusion of the cardiovascular studies; the contralateral (untreated) RVLM tissues were used as controls.

Effect of RVLM CB_1R Blockade on the Cardiovascular Effects of NAGly. In this experiment, we investigated the impact of CB_1R blockade, with the selective blocker SR141716 (Hohmann et al., 2005), on the BP response elicited by intra-RVLM NAGly because NAGly regulates AEA (CB_1R agonist) levels (Huang et al., 2001; Burstein et al., 2002). Four groups of conscious rats (n = 5 to 6 each) received one of the following intra-RVLM treatment combinations: 1) DMSO (solvent for SR141716) + vehicle, 2) SR141716 (0.1 μg) + vehicle, 3) DMSO + NAGly (1 μg), or 5) SR141716 + NAGly. SR141716 or DMSO was administered into the RVLM 30 minutes before NAGly or vehicle, and the dose of SR141716 was based on reported studies (Hohmann et al., 2005). DMSO was diluted in ACSF (1:16), and this DMSO-ACSF mixture had no effect on BP, which is consistent with our previous findings (Nassar et al., 2011). The BP and HR measurements continued for 30 minutes, after which the rats were euthanized and the brains were collected and stored at −80°C for subsequent biochemical studies.

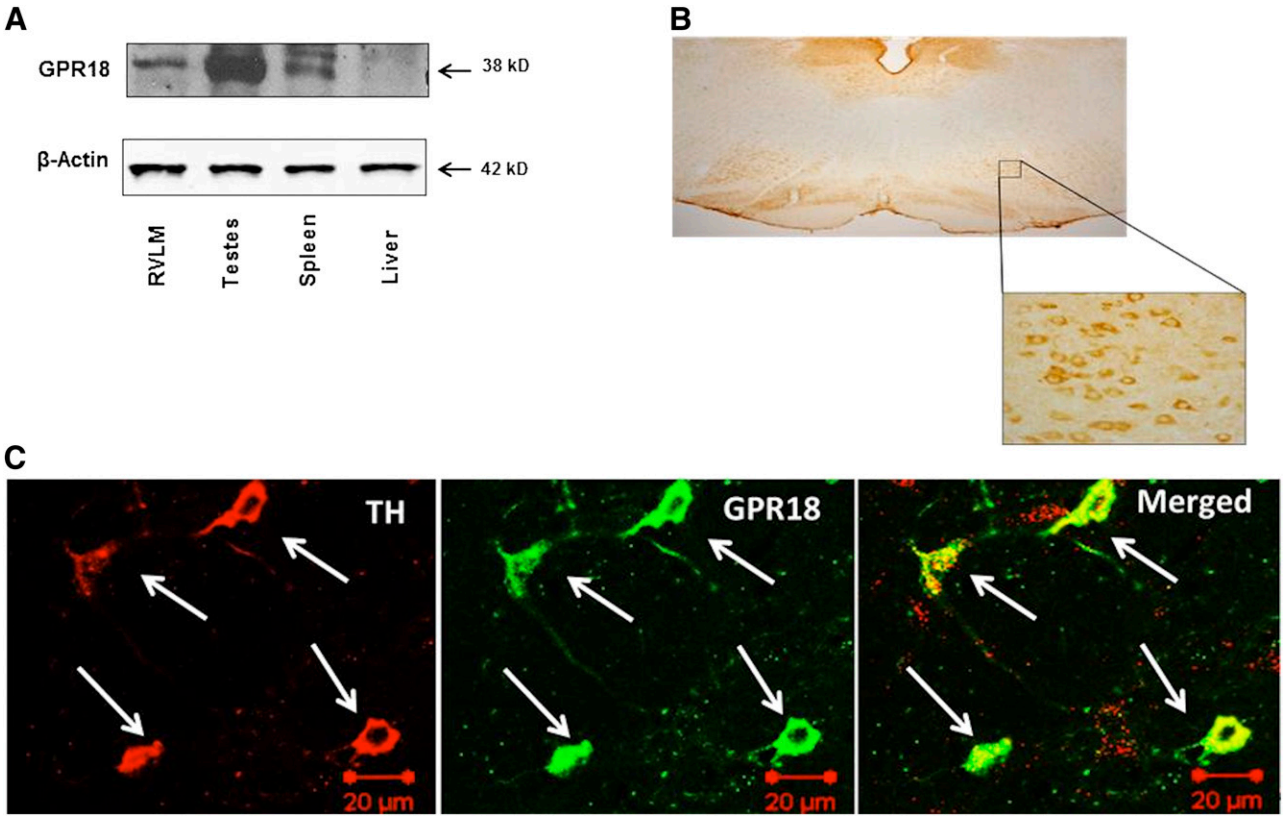

Fig. 1. (A) Expression of GPR18 (38 kDa) in the rat RVLM compared with expression in the testes and spleen (positive controls) and liver (negative control). (B) Immunohistochemical staining showing the expression of GPR18 in the RVLM of perfused naïve rat brains. (C) Dual-labeled immunofluorescence of perfused naïve rat brains showing coexpression of GPR18 and TH-expressing neurons in the RVLM.

Drugs. Abn CBD, NAGly, O-1918, and SR141716 were purchased from Cayman Chemical (Ann Arbor, MI). Methyl acetate, propranolol hydrochloride, atropine sulfate, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Adiponectin was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Sterile saline was purchased from B. Braun Medical (Irvine, CA). DMSO was used as the vehicle for SR141716. Methyl acetate was used as the vehicle for Abn CBD, O-1918, and NAGly and was tested in at least three animals without any significant changes in MAP and HR from the basal levels.

Data Analysis and Statistics. All values were expressed as mean ± S.E.M. change from their respective baselines. The dose-response curves were analyzed using repeated-measures analysis of variance using SPSS 16.0 statistical package for Windows (SPSS Inc., Chicago, IL) for differences in treatment trends. All other statistical analyses were done using a one-way or repeated-measures analysis of variance with Bonferroni's post hoc test and Student's *t* test, Prism 5.0 software (GraphPad Software Inc., San Diego, CA) was used to perform statistical analysis and $P < 0.05$ was considered significant.

Results

Expression of GPR18 in Tyrosine Hydroxylase Immunoreactive Neurons in the RVLM. Western blot (Fig. 1A) and immunohistochemical (Fig. 1B) findings revealed the expression of GPR18 in RVLM neuronal tissues. Positive and negative controls using tissues rich in (testis and spleen) or devoid of (liver) GPR18 verified the GPR18 findings (Fig. 1A). Further, dual-labeled immunofluorescence findings revealed the localization of GPR18 in tyrosine hydroxylase-ir neurons of the RVLM (Fig. 1C).

Activation of RVLM GPR18 Causes Hypotensive Response. These studies were conducted to elucidate the functional role of RVLM GPR18 in BP control. Compared with the vehicle (methyl acetate), intra-RVLM microinjection of the GPR18 agonist Abn-CBD caused dose-related reductions in BP along with tachycardic responses (Fig. 2, A and B). On the other hand, microinjection of the GPR18 antagonist O-1918 caused dose-dependent increases in BP along with bradycardic responses (Fig. 2, A and B). Prior autonomic blockade with atropine and propranolol (1 mg/kg each) had no significant effect on the dose-related reductions and elevations in BP caused by Abn CBD and O-1918, respectively, but fully abrogated the associated HR responses (Fig. 2, C and D). Notably, however, intra-RVLM microinjection of the endogenous GPR18 agonist NAGly caused small ($P > 0.05$) hypotensive (-5 ± 1 mm Hg; $n = 6$) and inconsistent HR responses (Fig.

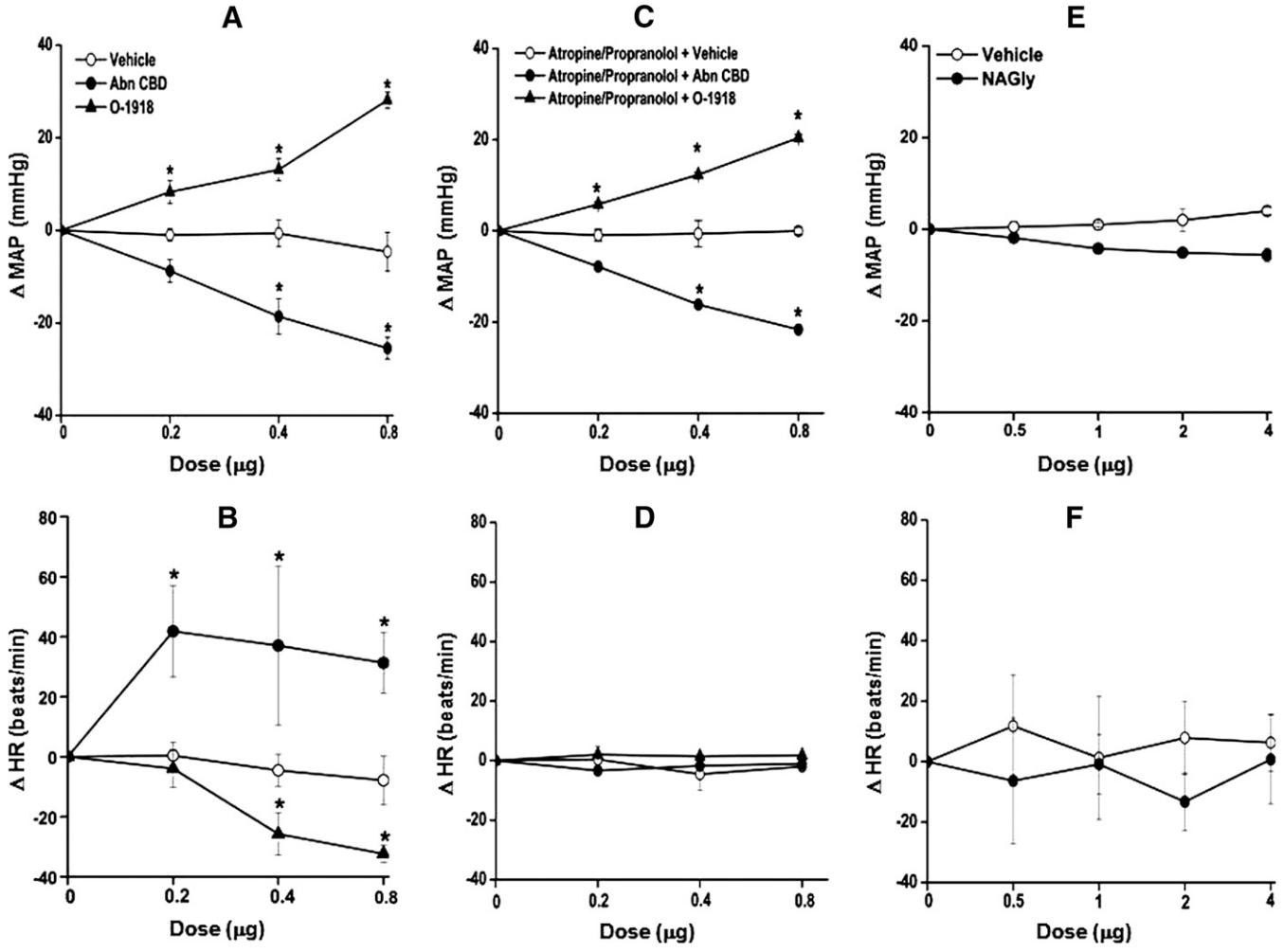

Fig. 2. Effect of intra-RVLM Abn CBD or O-1918 (0.2, 0.4, or 0.8 μg) (A and B) on MAP and HR in conscious male rats. (C and D) Effect of vagal and beta adrenergic blockade with atropine (1mg/kg) and propranolol (1 mg/kg), respectively, on the MAP and HR elicited by intra-RVLM Abn CBD or O-1918 (0.2, 0.4, or 0.8 μg). Effect of the endogenous ligand of GPR18, NAGly (0.5, 1, 2, or 4 μg) (E and F) on the MAP and HR. Values are mean ± S.E.M. of five or six observations. *$P < 0.05$ versus control (vehicle).

2, E and F). BP and HR values before Abn CBD or NAGly were not significantly different (Table 1). Compared with the vehicle control, RVLM GPR18 activation (Abn CBD) increased ADN and NO levels, whereas blockade (O-1918) reduced ADN levels (Fig. 3, A and B) in the RVLM.

Intra-RVLM ADN Reduces BP and Lowers ROS and Elevates NO Levels in RVLM. ADN caused dose-dependent reductions (–2 ± 2 to −12 ± 1 mm Hg; $n = 4$) in BP (Fig. 4A), but not in HR; however, slight increases in HR of the control group resulted in significant differences in HR at the 0.25- and 1-pmol doses (Fig. 4B). Compared with the control, ADN increased NOx (Fig. 4C) and reduced ROS (Fig. 4D) levels in the RVLM.

RVLM CB_1R Blockade Unmasks NAGly-Evoked Hypotension. This experiment was conducted to determine whether NAGly concomitant (indirect) activation of RVLM CB_1R masks the GPR18-mediated hypotensive response. Figure 5A shows colocalization of GPR18 and CB_1R in the presympathetic neurons of the RVLM of naïve rats, inferring potential interaction between the two receptors. Pharmacologic studies showed that selective RVLM CB_1R blockade with SR141716 (0.1 μg) caused modest but significant ($P < 0.05$) BP reduction (−8 ± 2 mm Hg; $n = 6$), whereas HR was not significantly changed (Fig. 5, B and C). Further, despite a gradual return of BP to baseline level within 30 minutes of SR141716 administration, prior CB_1R blockade significantly ($P < 0.05$) enhanced the hypotensive response (–11 ± 1 mm Hg; $n = 6$) caused by intra-RVLM NAGly (Fig. 5B). The effect of SR141716 at CB_1R lasts at least 2 hours as reported in a different model system (Jarbe et al., 2010). Therefore, NAGly-evoked hypotension was not a result of the additive hypotensive responses caused by NAGly and SR141716 (Fig. 5B). HR was not influenced by any of the treatments (Fig. 5C). Neurochemical findings showed that SR141716/NAGly treatment significantly increased ADN and NO levels (Fig. 3, A and B) and reduced ROS levels (Fig. 7A) in the RVLM compared with NAGly alone.

O-1918 Abrogated Abn CBD-Evoked Hypotension and Neurochemical Responses. To confirm the involvement of GPR18 in Abn CBD–evoked BP and RVLM neurochemical responses, Abn CBD was microinjected after the GPR18 antagonist O-1918. Intra-RVLM O-1918 (0.4 μg) abrogated ($P < 0.05$) the reduction in BP (Fig. 6, A and B) and the increases in RVLM ADN (Fig. 3A) and the NO level (Fig. 3B) caused by intra-RVLM Abn CBD (0.4 μg). The selected Abn CBD and O-1918 doses were based on the dose-response curves for these drugs (Fig. 2, A and B). Notably, at the time of administration of Abn CBD or its vehicle, the BP of O-1918 pretreated rats had declined toward pretreatment level (Fig. 6A), which rules out functional antagonism as a potential reason for O-1918 abrogation of the hypotensive effect of Abn CBD. Importantly, O-1918 blockade of GPR18 (Abn CBD)-mediated responses was evident for at least 1 hour (Caldwell et al., 2013). The HR responses were not significantly different among the different treatment groups during the observation period (Fig. 6B).

GPR18 Activation Reduces ROS Generation in the RVLM. Given the established link between ROS generation in the RVLM and sympathoexcitation/pressor response (Kishi

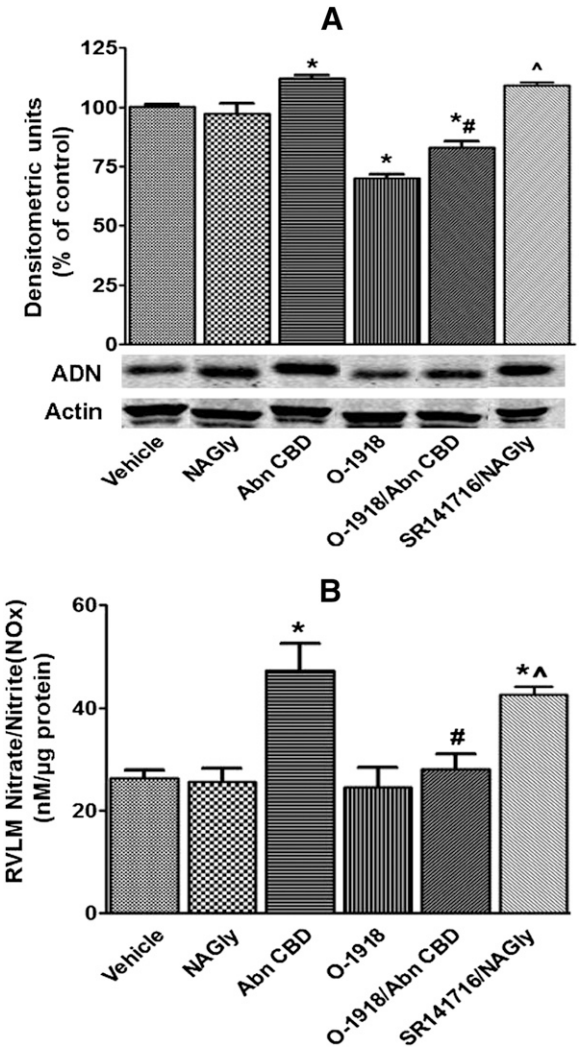

Fig. 3. Western blots and total nitrate/nitrite levels in the RVLM (NOx content; index of NO) showing the effects of NAGly (1 μg), Abn CBD (0.4 μg), O-1918 (0.4 μg) or O-1918 (0.4 μg)/Abn CBD (0.4 μg), or SR141716 (0.1μg)/NAGly (1 μg) treatment on ADN expression (A) and nitrate/nitrite (NOx) level (B) in the RVLM. Values are mean ± S.E.M. of five to six observations. *$P < 0.05$ vs. vehicle; #$P < 0.05$ vs. Abn CBD; ^$P < 0.05$ vs. NAGly values.

TABLE 1
Mean arterial pressure (MAP, mm Hg) and heart rate (HR, beats/min) values immediately before intra-RVLM administration of the GPR18 agonists or their vehicles

Group	*n*	MAP	HR
Vehicle	5	110 ± 5	364 ± 12
Abn CBD	6	100 ± 8	376 ± 9
NAGly	6	113 ± 2	327 ± 20
O-1918	6	108 ± 3	343 ± 7
Atropine/propranolol + vehicle	6	120 ± 5	353 ± 8
Atropine/propranolol + Abn CBD	6	120 ± 11	342 ± 24
Atropine/propranolol + O-1918	6	113 ± 5	340 ± 16
aDN	4	111 ± 5	336 ± 17
DMSO/vehicle	5	109 ± 7	325 ± 24
DMSO/NAGly	6	115 ± 5	356 ± 17
SR141716/vehicle	6	107 ± 6	352 ± 12
SR141716/NAGly	6	110 ± 8	360 ± 22
Vehicle/vehicle	5	108 ± 5	351 ± 9
Vehicle/Abn CBD	6	113 ± 4	362 ± 13
O-1918/vehicle	6	117 ± 6	345 ± 18
O-1918/Abn CBD	6	112 ± 7	366 ± 12

Abn CBD, abnormal cannabidiol [*trans*-4-[3-methyl-6-(1-methylethenyl) -2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol); DMSO, dimethylsulfoxide; ADN, adiponectin; NAGly, *N*-arachidonoyl glycine; RVLM, rostral ventrolateral medulla.

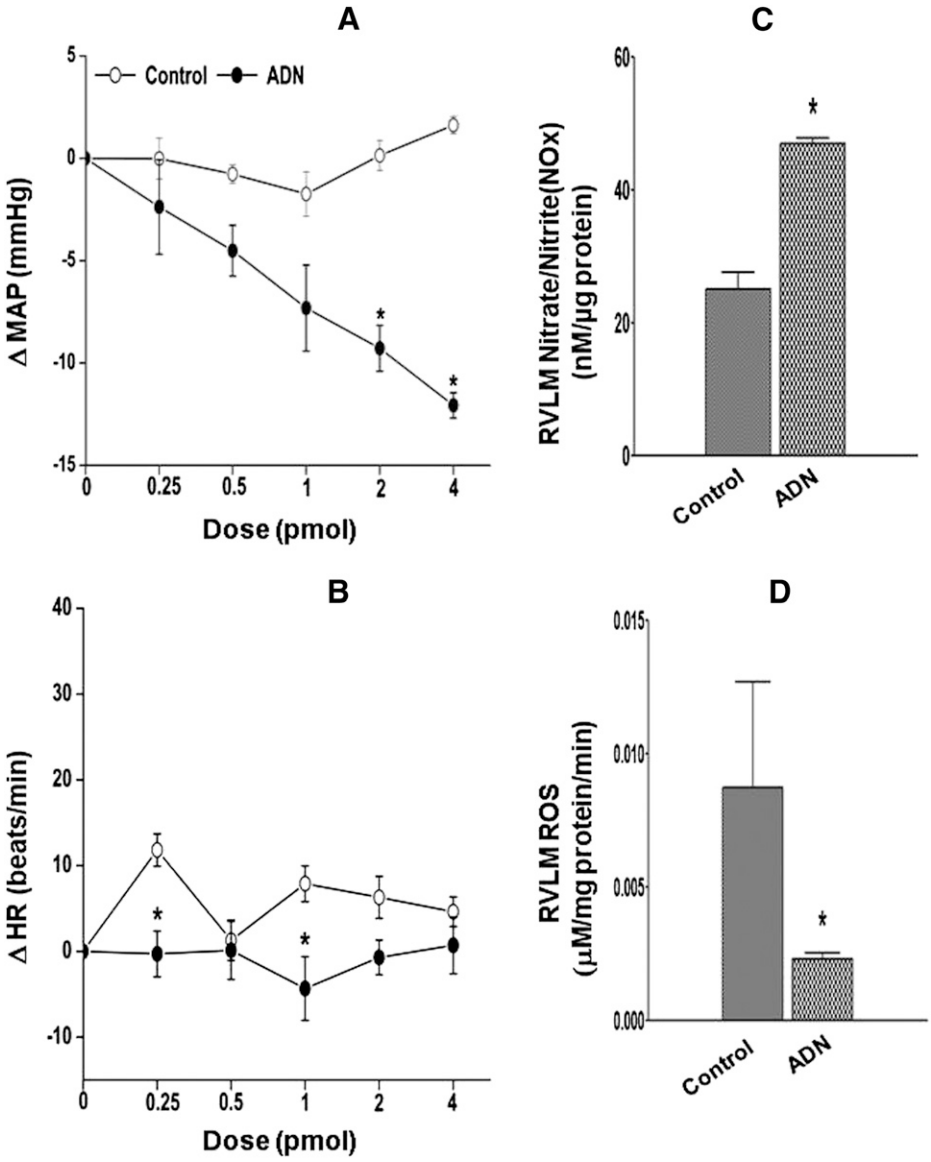

Fig. 4. Effect of intra-RVLM adiponectin, ADN (0.25, 0.5, 1, 2, and 4 pmol) (A, B) on MAP and HR in conscious male rats (n = 4), compared with values obtained from vehicle (ACSF)-treated rats (n = 3). Total nitrate/nitrite levels (NOx; index of NO) (C) and DCFH-DA measured ROS (D) in the RVLM after ADN microinjection compared with the corresponding values in the contralateral (control) RVLM. *$P < 0.05$ vs. contralateral RVLM levels.

et al., 2004), this experiment was conducted to determine the impact of GPR18 activation (Abn CBD) or blockade (O-1918) on the RVLM ROS level in neuronal tissues collected during the BP responses caused by these interventions. Compared with vehicle, Abn CBD significantly ($P < 0.05$) reduced (Fig. 7A), whereas O-1918 significantly ($P < 0.05$) increased, ROS levels in the RVLM (Fig. 7, A and B). Further, O-1918 abrogated the reduction in RVLM ROS caused by Abn CBD (Fig. 7A); these neurochemical responses paralleled the BP responses described already herein (Fig. 6, A and B).

Discussion

The following are the most important findings of the present study, which is the first to elucidate the function of RVLM GPR18 in conscious rats: 1) GPR18 is expressed in the TH-ir (presympathetic) neurons of the RVLM; 2) activation and blockade of RVLM GPR18 causes a dose-dependent reduction and elevation in BP, respectively; 3) GPR18 blockade (O-1918) abrogated Abn CBD–evoked hypotension; 4) concomitant CB_1R activation dampens the hypotensive response caused by the endogenous GPR18 ligand NAGly; 5) RVLM GPR18 activation increases ADN and NOx and reduces ROS levels in the RVLM, whereas its blockade produced the opposite neurochemical responses; 6) intra-RVLM ADN reduced BP and RVLM ROS and increased RVLM NOx. Collectively, the findings identify a novel sympathoinhibitory role for RVLM GPR18, mediated, at least partly, by reducing oxidative stress in the RVLM.

Although GPR18 mRNA is expressed in humans and mice (Vassilatis et al., 2003), we are the first to demonstrate GPR18 protein expression in the RVLM of rats (Fig. 1, A and B). Further, GPR18 is spatially located within the RVLM TH-ir neurons (Fig. 1C), which modulate the sympathetic activity (Sved et al., 2003; Guyenet, 2006), inferring GPR18 involvement in central control of sympathetic tone and BP. To elucidate the RVLM GPR18 functional role, we microinjected selective GPR18 agonist or antagonist into the RVLM of conscious rats. Abn CBD causes CB_1R/CB_2R-independent peripheral vasodilation (Randall et al., 2004; Johns et al.,

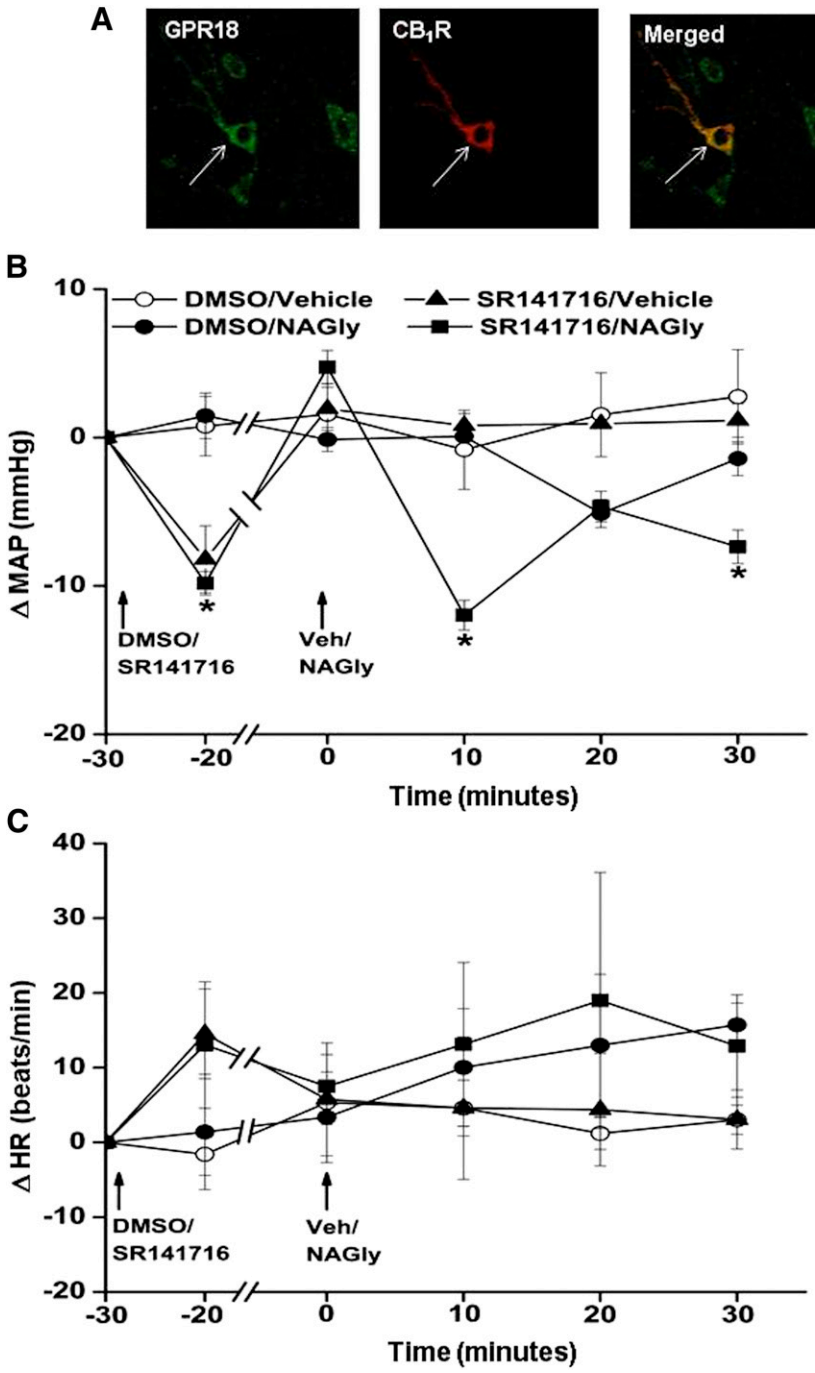

Fig. 5. (A) Dual-labeled immunofluorescence of perfused naïve rat brains showing coexpression of GPR18 and CB_1R in the RVLM neurons. Time-course changes in ΔMAP (B) and ΔHR (C) caused by intra-RVLM microinjection of DMSO/vehicle, DMSO/NAGly (1 μg), SR141716 (0.1μg)/vehicle, and SR141716 (0.1 μg)/NAGly (1 μg). The animals in each group received intra-RVLM microinjections of either DMSO (diluted 1:16 in ACSF) or SR141716 (0.1 μg) at −30 minutes followed by vehicle (methyl acetate) or NAGly (1 μg) at time "0". Pretreatment with SR141716 (CB1R blockade) uncovered NAGly (GPR18)-mediated hypotension. Values are means ± S.E.M. of five observations. $^*P < 0.05$ compared with the corresponding control value.

2007); in the latter study, Abn CBD was administered intravenously in anesthetized mice at much higher doses (30 mg/kg). Differences in the route of administration, dose, and anesthesia might explain the differences in the onsets and durations of the hypotensive responses and HR responses in the two studies. Here, we report the first evidence that intra-RVLM Abn CBD caused dose-dependent hypotensive response (Fig. 2A). This response was GPR18-mediated because it was abrogated (Fig. 6A) by O-1918, a selective GPR18 antagonist (McHugh et al., 2010). Further, intra-RVLM O-1918 elicited dose-related pressor response (Fig. 2A). Prior cardiac vagal and adrenergic blockade virtually abolished the HR, but not the BP, responses caused by GPR18 activation or blockade (Fig. 2, C and D), suggesting that the HR responses are mediated, at least partly, via cardiac baroreflex responses.

It is imperative to comment on the complexity of the observed HR responses. Whereas the reciprocal relationships between HR and BP responses elicited by Abn CBD or O-1918 (Fig. 2 and Supplemental Fig. 1S) support the involvement of the baroreceptors in the HR responses, it is notable that atropine and propranolol also block baroreceptor-independent cardiac responses. It is highly unlikely that Abn CBD produced direct chronotropic effects because it was microinjected into the RVLM in substantially lower doses than those used systemically (Johns et al., 2007). Nonetheless, it is possible that the central sympathoinhibitory effect of intra-

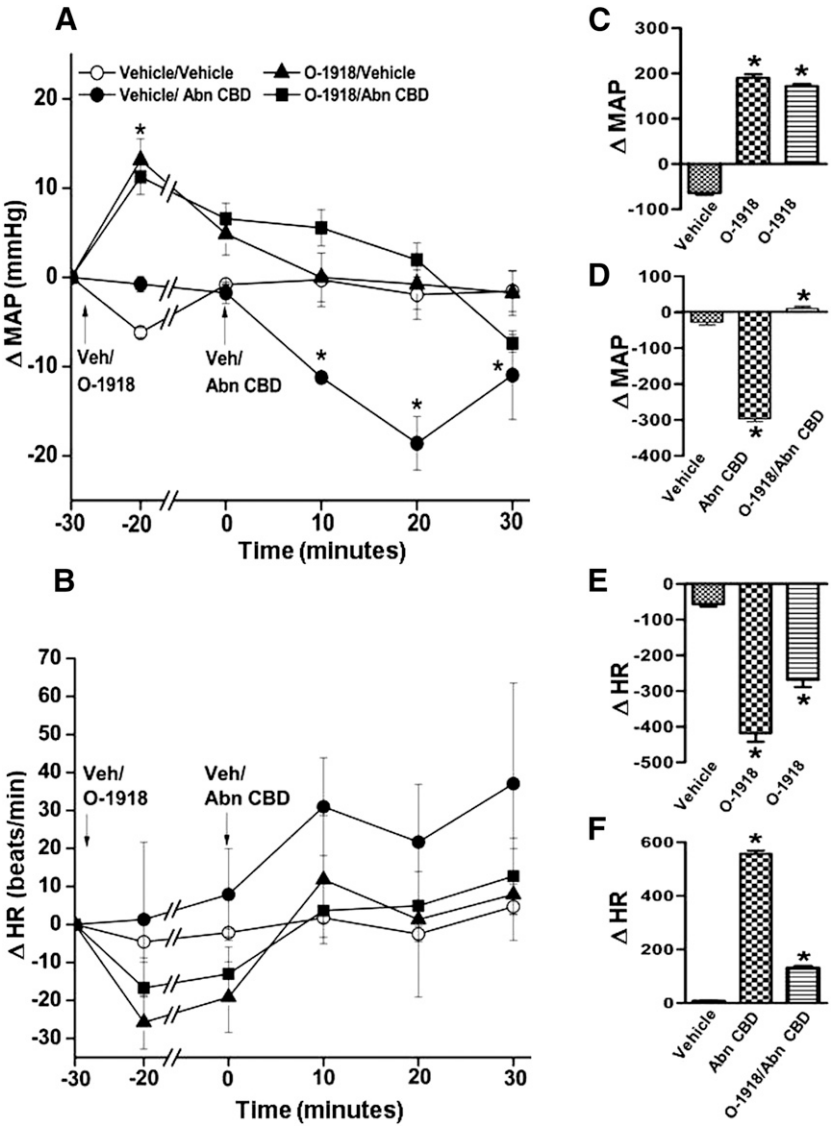

Fig. 6. (A and B) Time course of changes in MAP and HR after intra-RVLM microinjection of vehicle/Abn CBD (0.4 μg), O-1918 (0.4 μg)/vehicle, or O-1918 (0.4 μg)/Abn CBD (0.4 μg), compared with corresponding vehicle/vehicle values. The animals in each group received intra-RVLM microinjections of either vehicle (methyl acetate) or O-1918 (0.4 μg) at −30 minutes, followed by vehicle (methyl acetate) or Abn CBD (0.4 μg) at time 0. Pretreatment with O-1918 abrogated the hypotensive effect produced by Abn CBD and the associated tachycardic response. The bar graphs (C–F) depict the area under the curve (AUC) data generated from the time-course values over the pretreatment (−30 to 0 minutes) and treatment (0–30 minutes) periods. Compared with vehicle, the two groups that were pretreated with O-1918 exhibited significant elevations in BP and reductions in HR (Fig. 6, C and E). Treatment with Abn CBD caused significant reduction in BP and increase in HR, and these responses were abrogated in O-1918-pretreated rats (Fig. 6, D and F). Values are mean ± S.E.M. of five to six observations. *$P < 0.05$ versus vehicle.

RVLM Abn CBD might have dampened the tachycardic response. This possibility might explain the plateaued tachycardic response despite the dose-dependent hypotensive response caused by Abn CBD (Fig. 2) and the decline of the tachycardic response from its peak before the hypotensive response reached its nadir (Supplemental Fig. 1S). Collectively, these findings suggest that RVLM GPR18 exerts tonic restraining sympathoinhibitory influence on BP and that such central effect might contribute to the complexity of the observed HR responses.

The finding that intra-RVLM NAGly, the endogenous GPR18 ligand (Kohno et al., 2006), only modestly reduced BP (Fig. 2E) might 1) cast doubt about the biologic significance of RVLM GPR18 and 2) infer that Abn CBD mediated hypotension was a consequence of local redox changes in the RVLM rather than direct agonism at RVLM GPR18. Notably, NAGly can inhibit the enzyme fatty acid amide hydrolase, leading to increased AEA levels (Huang et al., 2001; Burstein et al., 2002). AEA may then activate central CB_1R, which mediates a sympathoexcitation/pressor response (Ibrahim and Abdel-Rahman, 2011) and ultimately counterbalances the GPR18-dependent reduction in BP caused by NAGly. In support of this notion are the following findings: 1) GPR18 and CB_1R are colocalized in RVLM neurons (Fig. 5A), which partly agrees with our findings that demonstrated CB_1R expression in RVLM TH-ir neurons (Ibrahim and Abdel-Rahman, 2011); 2) the ability of intra-RVLM SR141716 (CB_1R blockade) to lower BP (Fig. 5B) is consistent with a sympathoexcitatory/pressor function for central CB_1R (Ibrahim and Abdel-Rahman, 2011); 3) whereas NAGly alone had no effect on ADN, NOx, or ROS levels, prior CB_1R blockade (SR141716) uncovered the NAGly ability to increase ADN and NO (Fig. 3, A and B) and to reduce the ROS level (Fig. 7A) in the RVLM, along with lowering BP (Fig. 5B). These novel findings replicated the redox and BP effects of Abn CBD (Figs. 3 and 7A). Together, these data support the conclusion that the reductions in RVLM oxidative stress and BP are caused by Abn CBD direct agonism at GPR18 and suggest a functional role for GPR18-CB_1R interaction in the RVLM in modulating the local redox state and BP. It is imperative to comment on the pharmacologic perspectives of our study because the endogenous GPR18 ligand NAGly

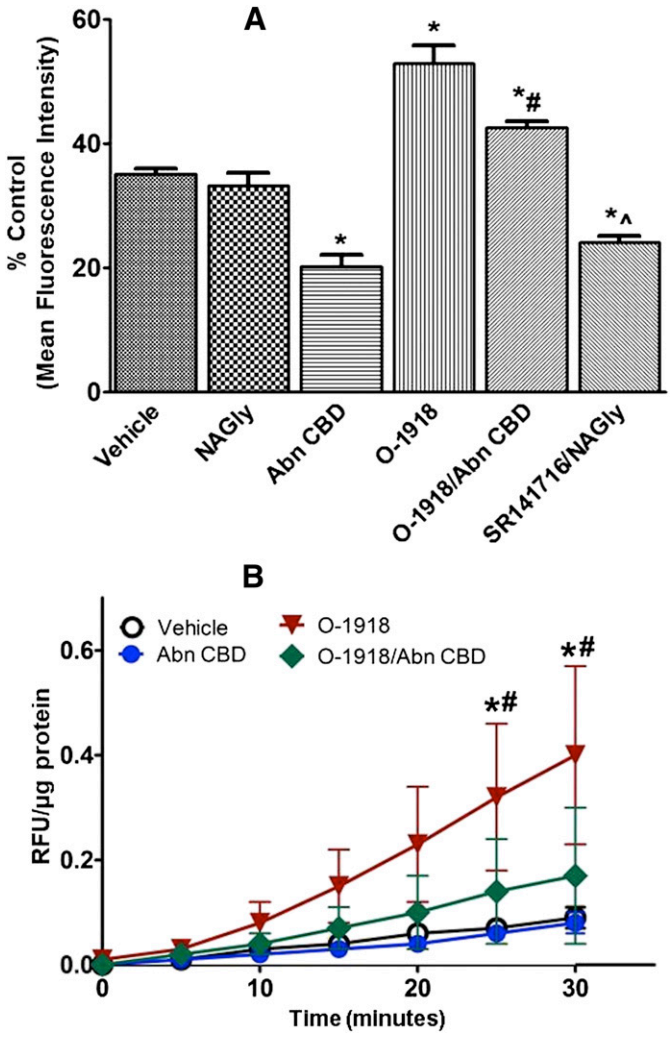

Fig. 7. (A) Effect of vehicle, NAGly (1μg), Abn CBD (0.4 μg), O-1918 (0.4 μg), O-1918 (0.4 μg)/Abn CBD (0.4 μg), and SR141716 (0.1μg)/NAGly (1 μg) on RVLM ROS levels shown by DHE staining visualized with confocal microscopy. Values are mean ± S.E.M. (n = five to six rats). *$P <$ 0.05 vs. vehicle values; #$P < 0.05$ vs. Abn CBD; ^$P < 0.05$ vs. NAGly values. (B) DCFH-DA measured ROS levels in terms of relative fluorescence units (RFU) in the RVLM after treatment with vehicle, Abn CBD (0.4 μg), O-1918 (0.4 μg), and O-1918 (0.4 μg)/Abn CBD (0.4 μg). Values of NAGly (1 μg) were not significantly different from the control and are not shown for clarity. Values are mean ± S.E.M. (n = five to six rats). *$P < 0.05$ versus vehicle values; #$P < 0.05$ vs. Abn CBD.

lowered BP only after blockade of CB_1R, which might infer that CB_1R blockade is required for uncovering the GPR18-mediated responses. Our present findings argue against generalizing this notion because the GPR18 agonist Abn CBD, which does not interact with CB_1R directly or indirectly (Jarai et al., 1999; Offertáler et al., 2003), lowered BP without prior CB_1R blockade.

It is important to comment on the complexity of RVLM NOS-derived NO in BP regulation because NO is implicated in GPR18-evoked hypotension (this study) and CB_1R-mediated hypertension (Ibrahim and Abdel-Rahman, 2012). Findings of the latter agree with a sympathoexcitatory role for RVLM NO (Chan et al., 2003). It is likely that the RVLM NO effect on BP depends on the source of NO and its effect on local sympathoinhibitory (GABA) and sympathoexcitatory (L-glutamate) neuromodulators. For example, although GABA inhibition is implicated in the NO-dependent CB_1R-mediated pressor response (Ibrahim and Abdel-Rahman, 2012), eNOS-derived NO mediates increases in the RVLM GABA level and hypotension (Kishi et al., 2001, 2002). More studies are warranted to delineate the mechanisms of the differential role of RVLM NO in modulating sympathetic activity/BP and to investigate the possibility that GPR18-dependent NO generation enhances RVLM GABA release/signaling in future studies.

A common anti-inflammatory role for ADN (Nanayakkara et al., 2012) and GPR18 (Vuong et al., 2008) infers a role for ADN in GPR18 signaling. We present the first evidence that GPR18 activation in the RVLM increases neuronal ADN (Fig. 3A), along with findings that support a functional role for ADN in GPR18 signaling because RVLM GPR18 blockade (O-1918) 1) reduced RVLM ADN (Fig. 3A) and elevated BP; and 2) abrogated the GPR18 (Abn CBD)-mediated BP and neurochemical responses (Figs. 3 and 6). Next, we show, for the first time, that ADN produced dose-dependent reductions in BP (Fig. 4A), increased RVLM NOx (Fig. 4C), and reduced RVLM ROS levels (Fig. 4D). The ADN doses were based on a reported microinjected dose of ADN in the area postrema (Fry et al., 2006). In the latter study, ADN caused a modest pressor response and inconsistent changes in HR. Differences in the neuroanatomic targets and use of anesthesia in the reported study might account for the differences in BP responses. Further, a very recent study (Song et al., 2013) showed that the active (globular) ADN fraction replicates ADN-evoked neuroprotection via a reduction in oxidative stress. Together, these findings implicate RVLM ADN in the GPR18-mediated reductions in neuronal oxidative stress (ROS) in the RVLM and BP.

We reasoned that the GPR18-mediated neurochemical responses, discussed in the preceding sections herein, would ultimately reduce BP via ROS reduction in RVLM because enhanced and suppressed ROS production in the RVLM leads to elevation and reduction in BP, respectively (Kishi et al., 2004; Hirooka, 2008). Using two different methods, we showed that activation of RVLM GPR18 reduced neuronal ROS, whereas its blockade increased neuronal ROS and abrogated the GPR18-mediated ROS reduction (Fig. 7). These redox findings, which paralleled the ADN (Fig. 3A) and BP (Fig. 6, A and B) responses, reinforce a well-established role for oxidative stress in RVLM neurons in sympathoexcitation and BP elevation. Further, the findings lend credence to our conclusion that ADN-dependent reduction in RVLM ROS plays a crucial role in GPR18-mediated hypotension.

In summary, the present study yields new insight into the role of the novel cannabinoid receptor GPR18 in central (RVLM) control of BP. We present the first evidence that RVLM GPR18 mediates reductions in oxidative stress and BP in conscious rats. The neurochemical findings suggest that increases in ADN and NO and reduced ROS production in the RVLM play a significant role in GPR18-mediated hypotension. In the RVLM, CB_1R serves a counterbalancing role against GPR18, which explains the negligible hypotensive response caused by the endogenous GPR18 ligand NAGly in our model system. Future studies are warranted to delineate the GPR18 signaling implicated in the neurochemical effects described in this study and to investigate the role of GPR18 signaling in hypertension. Such studies will advance our knowledge of the role of endocannabinoids in the neural control of BP and might lead to the development of novel antihypertensive drugs.

Acknowledgments

The authors thank Kui Sun for technical assistance.

Authorship Contributions

Participated in research design: Penumarti, Abdel-Rahman.

Conducted experiments: Penumarti.

Performed data analysis: Penumarti.

Contributed to the writing of the manuscript: Penumarti, Abdel-Rahman.

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