Anandamide-Mediated CB₁/CB₂ Cannabinoid Receptor-Independent Nitric Oxide Production in Rabbit Aortic Endothelial Cells

LaTronya McCollum,¹ Allyn C. Howlett,¹ and Somnath Mukhopadhyay

Neuroscience of Drug Abuse Research Program, Julius. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, North Carolina

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ABSTRACT

We have previously shown that the endocannabinoid anandamide and its metabolically stable analog (R)-methanandamide produce vasorelaxation in rabbit aortic ring preparations in an endothelium-dependent manner that could not be mimicked by other CB₁ cannabinoid receptor agonists (Howlett et al., 2002, 2004). Here, we show that (R)-methanandamide and abnormal cannabidiol stimulated nitric oxide (NO) production in rabbit aortic endothelial cells (RAEC) in a dose-dependent manner but that other CB₁ and CB₂ receptor agonists, such as cis-3R-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4R-3(3-hydroxypropyl)-1R-cyclohexanol (CP55940) and (R)-(−)-[2,3-dihydro-5-methyl-3-(4-morpholino)methyl]pyrrolo-[1,2,3-d,e]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone (WIN55212-2), failed to do so. CB₂ antagonists rimonabant [also known as SR141716; N-piperidin-1-yl]-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide and 6-methoxy-2-(4-methoxyphenyl)benzo[b]thien-5-yl]-4-cyanoxygenanthane (LY320135) and CB₂ antagonist N-[15]-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) failed to block (R)-methanandamide-mediated NO production in RAEC. However, anandamide receptor antagonist (−)-4-(3,4-trans-p-methylenedien-1,8)-yl-orcinol (O-1918) blocked (R)-methanandamide-mediated NO production in RAEC. Reverse transcriptase-polymerase chain reaction and Western blot analyses failed to detect the CB₂ receptor in RAEC, making this a good model to study non-CB₁ responses to anandamide. (R)-Methanandamide produced endothelial nitric-oxide synthase (eNOS) phosphorylation via the activation of phosphoinositide 3-kinase-Akt signaling. Inhibition of Gₛ signaling with pertussis toxin, or phosphatidylinositol 3-kinase-3 kinase activity with 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one (LY294002), resulted in a decrease in (R)-methanandamide-induced Akt phosphorylation and NO production. Results from this study suggest that in RAEC, (R)-methanandamide acts on a novel non-CB₁ and non-CB₂ anandamide receptor and signals through Gₛ and phosphatidylinositol 3-kinase, leading to Akt activation, eNOS phosphorylation, and NO production.

To date, two cannabinoid receptors have been identified: CB₁ cannabinoid receptors expressed predominantly in the brain (Howlett et al., 2002, 2004) and also in peripheral tissues, including the vascular endothelium (Liu et al., 2000; Kunos et al., 2002); and CB₂ cannabinoid receptors expressed in the cells of the immune and hematopoietic systems (Howlett et al., 2002; Klein et al., 2003) as well as in neurons (Van Sickle et al., 2005). Both CB₁ and CB₂ cannabinoid receptors are coupled to Gₛ proteins, and they inhibit

ABBREVIATIONS: CB₁, cannabinoid; MAPK, mitogen-activated protein kinase; PI3-kinase, phosphatidylinositol 3-kinase; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; RAEC, rabbit aortic endothelial cell(s); DAF-DA, 4-amino-5-methylamino-2,7’-difluorofluorescein diacetate; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; PCR, polymerase chain reaction; bp, base pairs; Abn-CBD, abnormal cannabidiol; CP55940, cis-3R-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4R-3(3-hydroxypropyl)-1R-cyclohexanol; WIN55212-2, [R(−)-][2,3-dihydro-5-methyl-3(4-morpholino)methyl]pyrrolo[1,2,3-d,e]-1,4-benzoazin-6-yl]-1-naphthalenylmethanone mesylate; rimonabant, SR141716, [N-piperidin-1-yl]-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; LY320135, [6-methoxy-2-(4-methoxyphenyl)benzo[b]thien-3-yl]-4-cyanoxygenanthane; SR144528, [N-[15]-endo-1,3,3,-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; O-1918, (−)-4-(3,4-trans-p-methylenedien-1,8)-yl-orcinol; RT-PCR, reverse transcriptase-polymerase chain reaction; HUVEC, human umbilical vein endothelial cell(s); LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one; PTX, pertussis toxin; HU-210, (−)-7-OH-Δ8-tetrahydrocannabinol-dimethylheptyl; DAF, 4-amino-dimethylamino-2,7’-difluorofluorescein.
adenylate cyclase (Howlett et al., 2004) and can also activate p42/44 mitogen-activated protein kinase (MAPK) (for review, see Howlett et al., 2004; Bouaboula et al., 1997) and the phosphatidylinositol 3-kinase (PI3-kinase)-Akt pathway (Liu et al., 2000).

The endocannabinoid anandamide and its metabolically stable analog (R)-methanandamide have been shown to produce hypotension and bradycardia in animal models (for review, see Kunos et al., 2000a,b, 2002) and to produce vasorelaxation in a number of vascular beds (Kunos et al., 2002; Mukhopadhyay et al., 2002). Several diverse mechanisms have been suggested to explain these physiological responses, including 1) anandamide serving as an endothelial-derived hyperpolarizing factor (Randall et al., 1997); 2) anandamide-stimulated CB1 cannabinoid receptors being sympathoinhibitory (for review, see Kunos et al., 2002); 3) anandamide-stimulated transient receptor potential vanilloid receptors, promoting release of mediators (Di Marzo et al., 2002); or 4) anandamide altering the function of gap junctions (Chaytor et al., 1999). There is growing evidence over the last few years that anandamide-mediated vasodilatation in the arterial (Mukhopadhyay et al., 2002), isolated mesenteric (for review, see Kunos et al., 2000a,b, 2002), and some other vascular preparations (O'Sullivan et al., 2004) is independent of CB1 or CB2 cannabinoid receptors and that vasodilatation is produced via the activation of a putative non-CB1/CB2 anandamide receptor. Findings from our laboratory showed that in rabbit aortic ring preparations (Mukhopadhyay et al., 2002), anandamide or (R)-methanandamide, acting on a non-CB1/CB2 endothelial site, produced vasorelaxation. In that study, anandamide- or (R)-methanandamide-induced vasorelaxation was sensitive to pertussis toxin, and it was blocked by NO synthase inhibitors, suggesting that anandamide might act on an endothelial non-CB1/CB2 G protein-coupled receptor to activate endothelial NO synthase (eNOS) to produce NO.

In the present study, the endothelial cell response to the endocannabinoid anandamide has been investigated using cultured rabbit aortic endothelial cells (RAEC) as a model system. We have pharmacologically characterized the anandamide response leading to NO production, and we investigated the molecular signaling mechanism of anandamide-mediated eNOS activation.

Materials and Methods

Cell Culture and Reagents. RAEC primary culture was established by S.M. in collaboration with Dr. Barry Chapnick (Saint Louis University School of Medicine, St. Louis, MO). Cells were maintained at 37°C under an atmosphere of 5% CO2 in phenol red-free endothelial basal media-A (Cambrex Bio Science, Walkersville, MD) containing 2% fetal bovine serum and growth factors for endothelial cells (Bullet kit; Cambrex Bio Science). Cells from passages three to five were used for experiments.

Measurement of NO. The production of NO was measured using 4-aminomethyl-2,7'-difluorofluorescein diacetate (DAF-DA) (Invitrogen, Carlsbad, CA), which has been reported previously to be linearly related to NO production (Montagnani et al., 2001). Endothelial cells were grown to 50% confluence on coverslips in 24-well plates. Cells were incubated with serum-free EBM-A for 16 h, and then they were loaded with the NO-reactive dye DAF-DA (final concentration, 5 μM) for 40 min at 37°C in the dark. DAF-DA is a cell-permeable compound that is converted to DAF-2 by intracellular esterases. DAF-2 forms a triazole derivative that emits light at 515 nm upon excitation at 489 nm in proportion to the amount of NO present inside the cells (Montagnani et al., 2001). After loading, cells were rinsed once with EBM-A and then treated with test compounds for 5 min in the dark at 37°C. For antagonist treatment, cells were pretreated with antagonist for 30 min or as indicated before addition of agonist compounds. After drug treatment, cells were rinsed with Dulbecco’s phosphate-buffered saline (PBS: 140 mM NaCl, 0.9 mM CaCl2, 0.49 mM MgCl2, 2.6 mM KCl, 8.0 mM KH2PO4, and 0.14 mM Na2HPO4, pH 7.4), fixed with 2% glutaraldehyde at 4°C, and then washed twice with PBS. This method of measuring intracellular NO using aldehyde fixatives (2% glutaraldehyde) with DAF-2 diacetate has been extensively investigated (Sugimoto et al., 2000; Takumida and Anniko, 2001) and has been found to be reliable for quantitation. The nuclei were stained with 4,6-diamidino-2-phenylindole, diactate (0.3 μM) (DAPI-dilactate). Slides were prepared using Prolong Mount (Invitrogen), and the deconvolved images were visualized at 20× magnification using a Nikon 600 inverted epifluorescence microscope and argon laser equipped with a Nikon DXM 1200 digital camera. Because the DAF-2 dye undergoes significant photobleaching, cells were maintained in the dark, and they were exposed to a light intensity of 20% for 3 s for each image captured. Background intensity was ascertained by treating the cells with the test compounds without DAF-DA, in which case, very little background intensity was observed. Fluorescence intensity was quantified using Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Silver Spring, MD), and all the images for DAF or DAPI were recorded at the same gain and exposure. The ratio of DAF to DAPI fluorescence intensity was used to normalize for the variation in cell number in a particular field. Each experiment was performed at least five times in duplicate.

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was isolated from confluent cultures of endothelial cells or tissues by using the Total RNA Isolation kit (Invitrogen). Total RNA (5 μg) was reverse-transcribed by random priming and incubation with 200 units of Moloney murine leukemia virus transcriptase at 37°C for 1 h. The resulting single-stranded cDNA (5 μl) was then subjected to 30 cycles of polymerase chain reaction (PCR) under the following conditions: denaturation at 95°C for 5 min; amplification cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C, with a 7-min extension at 72°C during the last cycle. Each PCR mixture (100 μl) contained the cDNA template, 1 μM primers, 200 μM dNTPs, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, and 2.5 mM Taq polymerase. The primers used to amplify the human CB1 receptor gene (Gérard, 1991) corresponded to the following sequences in transmembrane segment II, 5′-GCCCT-GGCGTGGCAGACACTCC-3′ (sense), and transmembrane segment IV, 5′-GACACGGCAGATCAATG-3′ (antisense). The expected size of the amplicons was 276 bp for the CB1 receptor. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide treatment.

Drug Treatment, Cell Lysate Preparation, and Western Blotting. RAEC were serum starved for 12 h, and then they were treated with (R)-methanandamide or abnormal cannabidiol (Abn-CBD; 4-(3-3,4-trans-p-methylenedioxy-1,8-yl)olivetol) or CB1 and CB2 agonist CP55940 or WIN55521-2 as indicated. After experimental treatments, RAEC were washed twice with ice-cold PBS, pH 7.4, and then they were scraped off the plate in lysis buffer [50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 1% (v/v) Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM Pefabloc (serine protease inhibitor), 1.04 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.08 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, and 14 μM cysteine protease inhibitor E-64 (Protease Inhibitor Cock- tail, Sigma-Aldrich, St. Louis, MO)]. Lysates were rotated for 1 h at 4°C, and insoluble material was removed by centrifugation at 10,000g for 5 min at 4°C. Equal amounts of the denatured proteins were loaded on the wells and subjected to SDS-polyacrylamide gel
(7.5%) electrophoresis (Mini Protean III, Bio-Rad, Hercules, CA). Proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, blocked for 1 h by incubation in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, and 100 mM NaCl) containing 5% (v/v) nonfat dry milk, followed by incubation (3 h at room temperature) with primary antibodies (1:1000). Antibodies used were mouse monoclonal anti-Akt, anti-phospho-Ser473-Akt, anti-eNOS, and anti-phospho-Ser1177-eNOS (BD Biosciences Transduction Laboratories, Lexington, KY). The polyvinylidene difluoride membranes were washed three times in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 h with goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:8000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Membranes were washed extensively with Tris-buffered saline/Tween 20 followed by water and then developed using enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Data Analysis. Quantitative values of fluorescence intensities and band densities were normalized and analyzed using GraphPad Prism 4.0. Log concentration-response data were analyzed by nonlinear regression analysis, and other data were compared with control using Student’s t test or one-way analysis of variance.

Results

Effects of Endocannabinoid and Cannabinoid Agonists on NO production in RAEC. The metabolically stable endocannabinoid analog (R)-methanandamide produced an increase in NO production in RAEC as detected by an increase in DAF-2 fluorescence intensity (Fig. 1A). Quantitative analysis (Fig. 1B) of the fluorescence intensity upon treatment with (R)-methanandamide revealed a dose-dependent (1 nM–1 μM) increase in NO production in RAEC, having an EC50 value (with 95% confidence intervals) of 9.4 ± 0.05 nM. We also tested the effect of Abn-CBD on NO production in RAEC. Abn-CBD does not bind to the CB1 receptor (Mo et al., 2004), but it has the ability to act as a relaxing factor in arterial preparations, and it has been implicated as a possible anandamide receptor-specific agonist (Ho and Hiley, 2003; Offertaler et al., 2003). Like (R)-methanandamide, Abn-CBD produced a significant increase in NO production compared with vehicle control in RAEC (Fig. 2, A and B). The concentrations of (R)-methanandamide and Abn-CBD used in this study were based on their ability to produce NO-dependent vasorelaxation in aortic ring preparations as determined in previous studies (Mukhopadhyay et al., 2002; Ho and Hiley, 2003; Offertaler et al., 2003).
then tested the effects of efficacious CB1 receptor agonists on NO production in RAEC. CB1 and CB2 agonists CP55940 and WIN55212-2 failed to stimulate NO production in RAEC (Fig. 2C). Both CP55940 and WIN55212-2 have been shown to be potent agonists of CB1 and CB2 receptors in cultured cells at less than 1 μM in different signal transduction assays that include inhibition of adenyl cyclase, activation of MAPK, and inhibition of Ca2+ channels (for reviews, see Howlett et al., 2002; Pertwee, 2005). Furthermore, in our previous study with rabbit aortic ring preparations (Mukhopadhyay et al., 2002), CP55940 and WIN55212-2 failed to produce vasorelaxation at the concentration (1 μM) used in this study. The failure of CB1 and CB2 agonists to stimulate NO production in RAEC suggests that (R)-methanandamide acts via a non-CB1/CB2 anandamide receptor in the RAEC to stimulate NO production. CP55940 or WIN55212-2 failed to inhibit NO donor. 3-Morpholinosydnonimine, HCl, and sodium nitroprusside mediated increases in the fluorescence intensity (data not shown), indicating that these compounds did not block the ability of the DAF assay to detect NO-mediated fluorescence in the cells.

To further examine the existence of a novel non-CB1/CB2 pharmacological target for anandamide in endothelial cells, CB1 antagonists rimonabant or LY320135 or CB2 antagonist SR144528 was used to block (R)-methanandamide-stimulated NO production in RAEC (Fig. 2C). Both rimonabant and LY320135 at 1 μM have been shown to be specific and potent antagonists for the CB1 receptor signal transduction in cultured cells when 1 μM of a potent agonist, such as CP55940, was used (Howlett et al., 2002; Pertwee, 2005). Neither rimonabant nor LY320135 was able to block (R)-methanandamide-stimulated NO production in RAEC (Fig. 3A). Quantitation of fluorescence intensity showed that CB1 antagonists did not produce a significant decrease in (R)-methanandamide-stimulated fluorescence intensity for NO production (Fig. 3B). Likewise, the CB2 antagonist SR144528 failed to block (R)-methanandamide-stimulated NO production in these cells (data not shown). Alternatively, non-CB1/CB2 anandamide receptor antagonist O-1918 significantly blocked anandamide-mediated NO generation in RAEC (Fig. 3C).

To determine the presence of CB1 receptors in the RAEC, both mRNA and protein content of the cells were examined. As shown in Fig. 4A, reverse transcriptase-PCR of RNA preparations isolated from human umbilical vein endothelial cells (HUVEC) (lane 1), and rat or rabbit brain (lanes 1 and 4), produced single discrete bands of the expected size (276 bp). However, no band was detectable using RAEC (lane 2). RNA without reverse transcription did not produce any amplicon (lane 5) indicating that there was no genomic DNA contamination. To determine the presence of CB1 receptor protein, Western blot analysis using anti-CB1 receptor antibody was performed with the HUVEC and RAEC cell membranes proteins. As shown in Fig. 4B, Western analysis of HUVEC membrane protein (17,000 g pellet) produced a strong band at the expected apparent molecular weight for the CB1 receptor monomer (lane 2). However, no bands were detected with RAEC cell membranes (lane 1). Collectively, results from RT-PCR and Western blot analysis indicate that RAEC cells do not
express detectable amounts of CB1 receptor. This would allow the use of these cells to investigate endothelial cell responses to the endocannabinoid anandamide that are not mediated by the CB1 receptor. We have also tested for the presence of the CB2 receptor in these cells using Western blot techniques, and we failed to detect any CB2 receptor protein in RAEC (data not shown).

**Signal Transduction Pathways for (R)-Methanandamide-Stimulated NO Production.** Results from previous studies have implicated the PI3-kinase-Akt pathway in eNOS activation and NO production. To characterize the role of the PI3-kinase pathway, we tested the effect of PI3-kinase inhibitor LY294002 on (R)-methanandamide-mediated NO production and Akt phosphorylation. As shown in Fig. 5A, LY294002 at 1 μM completely blocked (R)-methanandamide-stimulated NO production in RAEC. Quantitation of the fluorescence intensity showed a significant decrease (p < 0.05) in fluorescence intensity of (R)-methanandamide plus LY294002 compared with (R)-methanandamide alone (Fig. 5B). LY294002 failed to block NO donor 3-morpholinosydnonimine, HCl- and sodium nitroprusside-mediated increases in the fluorescence intensity (data not shown) under similar conditions, suggesting that LY294002 did not block the ability of the DAF assay to detect NO-mediated fluorescence in the cells. Western blot analysis (Fig. 5C) showed that (R)-methanandamide stimulated Akt phosphorylation (lane 4) over basal phosphorylation (lane 1). Pretreatment with the PI3-kinase inhibitor LY294002 (lane 3) blocked (R)-methanandamide-mediated Akt phosphorylation almost completely. Unlike (R)-methanandamide, the CB1 and CB2 agonist CP55940 failed to produce Akt phosphorylation (lane 2).

Pertussis toxin (PTX) acts to ADP-ribosylate Gi/o proteins, making the Gi unable to be stimulated by the receptor. To understand whether Gi proteins are involved in the NO production, we tested the effect of PTX treatment on (R)-methanandamide-mediated NO production in RAEC. Overnight (16-h) treatment with 100 ng/ml PTX completely attenuated the (R)-methanandamide-stimulated NO production in RAEC (Fig. 6A). Quantitation of fluorescence intensity indicated that (R)-methanandamide-stimulated NO production in RAEC was completely abrogated by PTX pretreatment.

**Fig. 4.** Determination of CB1 receptors in endothelial cells and other tissues. A, RT-PCR was performed using total RNA from rat brain (lane 1), RAEC (lane 2), HUVEC (lane 3), rabbit brain (lane 4), RNA from rabbit brain without reverse transcription (lane 5), and rabbit liver (lane 6 using specific primers for the human CB1 receptor gene. Lane 8 contained molecular weight standard (data not shown). B, Western blot detection of CB1 receptor in HUVEC and RAEC cell membranes. Western blot analysis was carried out with affinity-purified anti-CB1 receptor antibody as described in the text. Lanes 1 and 2 contained 20 μg of RAEC and HUVEC P2 membrane proteins, respectively. This is a representative blot of three separate experiments having similar results.

**Fig. 5.** Effect of PI3-kinase inhibitor on (R)-methanandamide-stimulated NO production in RAEC. DAF-DA-loaded cells were treated with PI3-kinase inhibitor LY294002 at 1 μM for 30 min at 37°C before treatment with 1 μM (R)-methanandamide and fixation as described previously under Materials and Methods. A, images of cells were from a representative experiment. B, in the presence of PI3-kinase inhibitor LY294002, there was no increase in fluorescence intensity upon (R)-methanandamide stimulation. The data were expressed as mean ± S.E.M. from three separate experiments for the treatments as indicated (48.7 ± 3.99, 9.07 ± 2.07, 78.8 ± 3.4, and 14.8 ± 1.13) and were analyzed using an unpaired Student’s t test (p < 0.0001). C, Western blot analysis of (R)-methanandamide stimulated Akt phosphorylation. Western blot analysis was carried out with anti-Akt and anti-phospho-Akt antibodies (1:1000). Equal amounts of protein (30 μg) were loaded: lane 1, control; lane 2, CP55940 (1 μM for 15 min); lane 3, (R)-methanandamide (1 μM for 15 min) plus PI3-kinase inhibitor LY294002 (1 μM); and lane 4, (R)-methanandamide (1 μM for 15 min). This is a representative blot of three separate experiments having similar results.
The existence of a non-CB<sub>1</sub>/non-CB<sub>2</sub> anandamide receptor has been suggested by our findings in rabbit aortic ring preparations (Mukhopadhyay et al., 2002) and by findings by others in rat mesenteric arteries (for review, see Kunos et al., 2000a,b 2002; Offertaler et al., 2003) and other vascular preparations or in whole animals (for review, see Kunos et al., 2000a,b, 2002). A preliminary report that an orphan G protein-coupled receptor, GPR55, is capable of binding to cannabinoid ligands suggests that alternative receptors for anandamide may exist (Brown et al., 2005; Sjögren et al., 2005). Pharmacological characterization of the signal transduction response in cells that endogenously express the protein is the best argument for the existence of a putative receptor. Based upon the agonist and antagonist response profile, neither GPR55, nor the novel splice variant of the CB<sub>1</sub> receptor, seem to be obvious candidates for the endothelial receptor described here. The major caveat to dissecting the CB<sub>1</sub> response apart from that of the anandamide receptor is 1) the lack of specific agonist ligands for the anandamide receptor; and 2) the expression of CB<sub>1</sub> receptors in most of the vascular beds and endothelial cells (for review, see Kunos et al., 2000a,b, 2002). Thus, the distinction of CB<sub>1</sub> receptor responses from those of a novel anandamide receptor remains a challenge to investigators. In our earlier findings with rabbit aortic ring preparations, we described the existence of a novel non-CB<sub>1</sub>/non-CB<sub>2</sub> endothelial anandamide receptor (Mukhopadhyay et al., 2002). In that study, we described the failure of potent CB<sub>1</sub> and CB<sub>2</sub> agonists to mimic the anandamide-mediated vasorelaxation in rabbit aortic ring preparations. The current study used the cellular model of RAEC to study the pharmacology and mechanism of action of (R)-methanandamide. Here, using RT-PCR and Western blot analyses, we showed that RAEC do not express CB<sub>1</sub> receptor message or protein. This finding makes RAEC an excellent model to study the non-CB<sub>1</sub>/non-CB<sub>2</sub> anandamide receptor response relative to various cellular responses, including NO production and angiogenesis.

The failure of potent CB<sub>1</sub> and CB<sub>2</sub> agonists CP55940 and WIN55212 to produce NO in RAEC supports our previous findings in rabbit aortic ring preparations (Mukhopadhyay et al., 2002), in which CB<sub>1</sub>and CB<sub>2</sub> agonists WIN55212 and desacytylevorantradol failed to produce vasorelaxation. Abn-CBD is a synthetic analog of the behaviorally inactive plant-derived cannabinoid cannabidiol. Abn-CBD was first reported to be an inactive cannabinoid in neurobehavioral paradigms used to screen cannabinoids, but it was able to cause hypotension in dogs (Adams et al., 1977). Abn-CBD is a cannabinoid analog of the behaviorally inactive plant-derived cannabinoid cannabidiol. Abn-CBD was first reported to be an inactive cannabinoid in neurobehavioral paradigms used to screen cannabinoids, but it was able to cause hypotension in dogs (Adams et al., 1977). Abn-CBD is a cannabinoid analog of the behaviorally inactive plant-derived cannabinoid cannabidiol. Abn-CBD was first reported to be an inactive cannabinoid in neurobehavioral paradigms used to screen cannabinoids, but it was able to cause hypotension in dogs (Adams et al., 1977). Abn-CBD is a cannabinoid analog of the behaviorally inactive plant-derived cannabinoid cannabidiol. Abn-CBD was first reported to be an inactive cannabinoid in neurobehavioral paradigms used to screen cannabinoids, but it was able to cause hypotension in dogs (Adams et al., 1977). Abn-CBD is a cannabinoid analog of the behaviorally inactive plant-derived cannabinoid cannabidiol. Abn-CBD was first reported to be an inactive cannabinoid in neurobehavioral paradigms used to screen cannabinoids, but it was able to cause hypotension in dogs (Adams et al., 1977).
ability of Abn-CBD and (R)-methanandamide to produce NO in RAEC supports the existence of an anandamide and Abn-CBD-sensitive receptor in this cell. These findings corroborated those of Kunos et al. (2000a,b, 2002) in mesenteric vascular bed preparations, except that the Abn-CBD-mediated vasodilatory response was not inhibited by N^\*-nitro-L-arginine methyl ester, suggesting that endothelial NO was not the only factor involved in the Abn-CBD-mediated vasodilation.

Further support for the involvement of a non-CB1/non-CB2 anandamide receptor in (R)-methanandamide-mediated NO production in RAEC resulted from the experiments with antagonists. CB1 and CB2 receptor antagonists failed to block (R)-methanandamide-induced NO production, whereas non-CB1/CB2 antagonist O-1918 significantly blocked methanandamide-mediated NO production in RAEC. Similar to our findings, Offertaler et al. (2003) also reported that O-1918 blocked Abn-CBD-mediated vasorelaxation in endothelium-intact mesenteric arteries and also blocked Abn-CBD-mediated hypotensive effect. In the rabbit aortic ring preparations, rimonabant failed to block (R)-methanandamide-mediated endothelium-dependent vasorelaxation at 1 μM, and it only partially (20%) blocked endothelium-independent vasorelaxation at a higher concentration of 20 μM (Mukhopadhyay et al., 2002). This was not consistent with the inhibition of a CB1 receptor, and we have discussed this in the previous study (Mukhopadhyay et al., 2002). In CB1 wild-type C57BL/6 mice, anandamide or the cannabinoid agonist HU-210 mediated prolonged hypotension, which was blocked by pretreatment with 3 mg/kg rimonabant. These effects were completely absent in CB1 (−/−) C57BL/6 mice (for review, see Kunos et al., 2000a,b, 2002). In the buffer-perfused mesenteric vascular preparations from transgenic CB1 (−/−) mice, Abn-CBD evoked long-lasting vasodilation, which was significantly inhibited by rimonabant at 1 to 5 μM. In mesenteric preparations from CB1 (−/−) and CB2 (−/−) C57BL/6 mice, anandamide and Abn-CBD caused vasodilation similar to that in preparations from CB1 (−/−) C57BL/6 mice (for review, see Kunos et al., 2000a,b, 2002), indicating that anandamide and Abn-CBD act at a vascular site other than CB1 or CB2 receptors.

A non-CB1/non-CB2 site was reported to exist on glutamatergic terminals in the mouse hippocampus, where its activation by cannabinoids inhibited glutamatergic transmission (Hajos et al., 2001). The site in the hippocampus was susceptible to inhibition by rimonabant, but it differed from the endothelial cell response in that it could be activated by the synthetic cannabinoid WIN55212-2 (Hajos et al., 2001). Breivogel et al. (2001) reported an anandamide- and WIN55212-2-sensitive, non-CB1/non-CB2 site that could stimulate guanosine 5’-O-(3-thio)triphosphate binding to G proteins in brain membranes from CB1 (−/−) C57BL/6 mice (Breivogel et al., 2001), and this target failed to be blocked with rimonabant. Another WIN55212-2-sensitive but rimonabant-insensitive, non-CB1/non-CB2 site identified in astrocytes led to inhibition of CAMP production (Sagan et al., 1999). Because the site we are describing in this article, or described early in vascular preparations (Mukhopadhyay et al., 2002), is insensitive to WIN55212-2, it is very likely distinct from the non-CB1 sites described in the central nervous system above.

Endothelium-derived NO is produced by eNOS, which exhibits a complex regulatory mechanism. eNOS possesses oxidizing and reducing domains that are attached by a calmodulin binding site (for review, see Fulton et al., 2001). Although eNOS was originally regarded as a strictly Ca^{2+}/calmodulin-dependent enzyme, there is a growing body of evidence that eNOS is also activated in a Ca^{2+}/calmodulin-independent manner when stimulated with agonists such as adiponectin (Hattori et al., 2003) or insulin (Hartell et al., 2005), or by mechanical shear-force (Fleming et al., 1999; Boo, 2006). Furthermore, several other positive regulators (90-kDa heat shock protein, dynamin-2, signaling kinase Akt, and MAPK) and negative regulators (caveolin and NO synthase interacting protein) have also been implicated in eNOS activity.

Phosphorylation of eNOS plays a vital role in the regulation of eNOS (for review, see Shaul, 2002). eNOS is primarily phosphorylated on Ser residues, and to a lesser extent, on Tyr or Thr residues. Multiple protein kinases, including cyclic AMP-dependent protein kinase (Michelli et al., 2001; Boo, 2006), PI3-kinase-Akt (Hartell et al., 2005), and MAPK (Anter et al., 2005), have been implicated in eNOS phosphorylation. Bernier et al. (2000) have documented that the vasodilator bradykinin activated eNOS by Ser1177 phosphorylation via the activation of MAPK. Our evidence that (R)-methanandamide produced an Akt-mediated phosphorylation at Ser1177 is in agreement with the results reported by Sessa and colleagues in bovine aortic endothelial cells stimulated with sphingosine-1-phosphate (Igarashi and Michel, 2001; Lin et al., 2003). Protein kinase C signaling acts in opposition by phosphorylating Thr^{195} residues and promoting phosphatase-mediated Ser^{1177} dephosphorylation (Li et al., 2004). Our data are consistent with eNOS activation via a PI3-kinase-Akt pathway that involves a mechanism requiring G_{αi} activation.

The findings reported in this study are important to our understanding of the endocannabinoid signaling system in endothelial cells. Anandamide is biosynthesized throughout the body, and it is beginning to be understood that CB1 receptors are also present in many cell types outside of the central nervous system. If we can characterize the pharmacology of alternative targets for the action of anandamide, we might be able to generate selective agonists directed at therapeutically beneficial responses to anandamide, such as vasodilation.

References
**Address correspondence to:** Dr. Somnath Mukhopadhyay, Neuroscience of Drug Abuse Research Program, J. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, 700 George St., Durham, NC 27707. E-mail: smukhopadhyay@nccu.edu