Cardiovascular Pharmacology

Time-dependent vascular actions of cannabidiol in the rat aorta

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A B S T R A C T

We have shown that the major active agent of Cannabis sativa, Δ2-tetrahydrocannabinol, activates peroxisome proliferator-activated receptor gamma (PPARγ). O’Sullivan, S.E., Tarling, E.J., Bennett, A.J., Kendall, D.A., Randall, M.D., 2005c. Novel time-dependent vascular actions of delta9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. Biochem. Biophys. Res. Commun. 337, 824–831. The aim of the present study was to investigate whether another pharmacologically active phytocannabinoid, cannabidiol, similarly activates PPARγ. Functional vascular studies were carried out in rat aortae in vitro by myography. PPARγ activation was investigated using reporter gene assays, a PPARγ competition-binding assay and an adipogenesis assay. Cannabidiol caused time-dependent (over 2 h) vasorelaxation of pre-constricted aortae, sensitive to PPARγ antagonism (GW9662, 1 µM) and super oxide dismutase inhibition. The vascular effects of cannabidiol were not affected by endothelial denudation, nitric oxide synthase inhibition, pertussis toxin, cannabinoid CB1 or cannabinoid CB2 receptor antagonism, or capsaicin pre-treatment. When aortae were contracted with U46619 in a Ca²⁺−free buffer, vasorelaxation to cannabidiol was substantially reduced. Furthermore, cannabidiol (1–30 µM) inhibited the contractile response to the re-introduction of Ca²⁺. In a reporter gene assay, cannabidiol increased the transcriptional activity of PPARγ. Cannabidiol was also found to bind to PPARγ and stimulate the differentiation of 3T3-L1 fibroblasts into adipocytes, a PPARγ-mediated response. These results show that cannabidiol binds to and activates PPARγ, which partially underlies the time-dependent vascular effects of cannabidiol. However, cannabidiol-induced vasorelaxation in the rat isolated aorta appears to be largely due to calcium channel inhibition.

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1. Introduction

Cannabidiol is a major component of the cannabis plant, Cannabis sativa, but lacks the psychotropic effects of the better known active agent, Δ2-tetrahydrocannabinol (THC). Cannabidiol is anxiolytic (Moreira et al., 2006; Zuardi et al., 2006; Restel et al., 2006) and may antagonise the psychotropic effects of THC in the clinically available cannabis-based medicine, Sativex (Russo and Guy, 2006). In addition to its behavioural effects, cannabidiol has been shown to be anti-inflammatory (Costa et al., 2007; Esposito et al., 2007), antioxidant (García-Arencibia et al., 2007), neuroprotective (El-Remessy et al., 2006), a potent inhibitor of cancer cell growth (Ligresti et al., 2006) and is beneficial in diabetes (El-Remessy et al., 2006; Weiss et al., 2006). However, the molecular targets for cannabidiol are still unclear. Cannabidiol is reported to have a low affinity for both of the ‘classical’ G-protein-coupled cannabinoid receptors, cannabinoid CB1 and cannabinoid CB2 (McPartland et al., 2007), although it has been shown to antagonise both cannabinoid CB1 and cannabinoid CB2 receptors in the low nanomolar range (Thomas et al., 2007). Cannabidiol is also suggested to be an antagonist of the GPR55 receptor (Ryberg et al., 2007). Peroxisome proliferator-activated receptors (PPARs) belong to a family of nuclear receptors of which there are three isotypes: α, δ and γ (Ferré, 2004). PPARs heterodimerise with the retinoid X receptor, and bind to DNA sequences called PPAR response elements (PPREs), which lead to the transcription of target genes upon ligand activation. PPARs are primarily involved in the regulation of metabolism and energy homeostasis. For example, agonists of the PPARδ isoform improve insulin sensitivity and are used in the management of type 2 diabetes (Ferré, 2004; Rangwala and Lazar, 2004). In addition, PPARγ agonists have been shown to have positive cardiovascular effects, which include increased availability of nitric oxide, reductions in blood pressure and attenuation of atherosclerosis (Bishop-Bailey, 2000; Hsueh and Brummer, 2004). Some of the beneficial effects of PPARγ ligands are brought about by anti-inflammatory actions, including inhibition of pro-inflammatory cytokines, increasing anti-inflammatory cytokines, and inhibition of inducible nitric oxide synthesis.
synthase (iNOS) expression (see Széles et al., 2007 for a recent review).

PPARs have a large ligand binding pocket and are pharmacologically promiscuous, being activated by a number of natural and synthetic ligands including, as reported recently, cannabinoids (see O'Sullivan, 2007 for a recent review). Specifically, it has been reported that ajulemic acid, an analogue of a THC metabolite, binds to and increases the transcriptional activity of PPARγ with anti-inflammatory actions (Liu et al., 2003). Similarly, the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) have anti-inflammatory actions sensitive to PPARγ antagonism (Rockwell and Kaminski, 2004; Rockwell et al., 2006). Anandamide has also been shown to directly bind to PPARγ (Bouaboula et al., 2005; Gasperi et al., 2007). We recently showed that THC increases the transcriptional activity of PPARγ and leads to vascular responses that are inhibited by a PPARγ antagonist (O'Sullivan et al., 2005c, 2006). As many of the effects of cannabidiol treatment are consistent with those of PPARγ activation, and since other cannabinoid compounds have been shown to activate PPARγ, the possibility exists that PPARγ activation might underlie some of the pharmacological effects of cannabidiol.

In vitro, abnormal cannabidiol, a synthetic analogue of cannabidiol, causes vasorelaxation of rat isolated mesenteric arteries via the putative ‘endothelial’ cannabinoid receptor, potassium channel activation and calcium channel blockade (Offertäler et al., 2003; Ho and Hiley, 2003). In vivo, abnormal cannabidiol causes hypotension and mesenteric vasodilatation in wild-type mice and in mice lacking both cannabinoid CB1 and cannabinoid CB2 receptors (Járai et al., 1999). However, to date, the vascular responses to cannabidiol (as opposed to abnormal cannabidiol) remain uninvestigated. We have previously shown that THC causes time-dependent, endothelium-dependent, PPARγ-mediated vasorelaxation of the rat isolated aorta (O'Sullivan et al., 2005c). Therefore, the aim of the present study was to investigate whether similar time-dependent, PPARγ-mediated vasorelaxation to cannabidiol occurs in the rat aorta.

Our studies have shown for the first time that cannabidiol causes vasorelaxation of the rat isolated aorta, partly mediated by PPARγ. We have demonstrated that cannabidiol increases the transcriptional activity of PPARγ, binds to PPARγ and causes a PPARγ-mediated response, adipogenesis (Mueller et al., 2002). These data confirm that cannabidiol is a PPARγ ligand, and suggest that PPARγ activation may underlie some of the pharmacological effects of cannabidiol.

2. Methods and materials

2.1. In vitro vascular studies

Male Wistar rats (250–350 g) were stunned by a blow to the back of the head and killed by cervical dislocation under Schedule 1 to the Animals Act 1986 (Scientific Procedures). The aortae were removed rapidly and placed into cold modified Krebs–Henseleit buffer (composition, mM: NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 2, and o-glucose 10). The aortae were dissected free of adherent connective and adipose tissue and cut into rings 3–4 mm long, and mounted on fixed segment support pins using the Multi Myograph system (Model 610 M, Danish Myo Technology, Denmark). Once mounted, all vessels were kept at 37 °C in modified Krebs–Henseleit buffer and gassed with 5% CO2 in O2. The aortae were stretched to an optimal passive tension of 9.8 mN tension (O'Sullivan et al., 2005c). Vessels were allowed to equilibrate and the contractile integrity of each was tested by its ability to contract to 60 mM KCl by at least 4.9 mN. Vessels were contracted with a combination of U46619 (10–100 nM, a thromboxane prostanoid receptor agonist), and the α-adrenoceptor agonist methoxamine (1–5 µM) to increase tension as previously described (O'Sullivan et al., 2005c). When a stable contraction was maintained, the vasorelaxant effect of a single concentration of cannabidiol or vehicle control (0.1% ethanol) on induced tone was assessed as the reduction in tone over time. For each experimental protocol, vehicle control and cannabidiol-treated experiments were performed in adjacent segments of the same artery. Net relaxation was calculated as the vasorelaxant effect of cannabidiol minus the vasorelaxant effect of vehicle (in adjacent aortic segments) over time. In some experiments, the vascular effects of cannabidiol were investigated in un-contracted vessels.

To assess the contribution of PPARγ activation, some experiments were performed in the presence of the PPARγ antagonist GW9662 (1 µM) added 10 min prior to pre-contraction. To investigate the role of endothelium-derived relaxing factors in the time-dependent vasorelaxation to cannabidiol, some vessels were denuded of their endothelium by abrasion with a human hair. The role of endothelium-derived nitric oxide (NO) was investigated using the nitric oxide synthase inhibitor, Nω-nitro-arginine methyl ester (L-NAME, 300 µM, present throughout, O'Sullivan et al., 2005c). To establish a potential role for superoxide dismutase (SOD) activity, some experiments were performed in the presence of the SOD inhibitor diethyldithiocarbamate (DETA, 3 mM), added 30 min prior to pre-contraction of arteries (O'Sullivan et al., 2005c).

To assess any possible contribution of cannabinoid receptor activation, some experiments were performed in the presence of the cannabinoid CB1 receptor antagonist, AM251 (1 µM), or the cannabinoid CB2 receptor antagonist, AM630 (1 µM), both added 10 min before contracting vessels. To assess if cannabidiol acts at a G(i/o)-protein-coupled receptor, some vessels were incubated for 2 h with 200 ng/ml pertussis toxin (PTX, O'Sullivan et al., 2005b), and vasorelaxation to cannabidiol or vehicle assessed. The involvement of TRPV1 receptors on sensory nerves was assessed by incubating vessels for 1 h with the TRPV1 agonist capsaicin (10 µM) to deplete the sensory nerves of vasoactive neurotransmitters (Zygmont et al., 1999; O'Sullivan et al., 2005a).

To investigate the involvement of K+ channel activation in vasorelaxation to cannabidiol, some experiments were performed in vessels pre-contracted with a high-K+ (60 mM) buffer to inhibit potassium efflux. To test whether cannabidiol causes vasorelaxation in arteries in Ca2+-free conditions, some vessels were contracted with U46619 in Ca2+-free Krebs and the vasorelaxant effects of cannabidiol investigated. To investigate the effects of THC on Ca2+ influx, concentration–response curves to calcium chloride (CaCl2, 1 µM to 100 mM) were performed in the presence and absence of cannabidiol (1, 10 and 30 µM, 10 min incubation). The vessels were first allowed to equilibrate in Ca2+-free buffer, and were then bathed in Ca2+-free, high-K+ buffer. 10 min after adding cannabidiol, a concentration–response curve to re-introduced CaCl2 was constructed.

2.2. Cell culture

Human embryonic kidney (HEK293) cells and 3T3L1 fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum with antibiotics (penicillin, streptomycin and gentamicin) in 5% CO2.

2.3. Transactivation assay

Confluent HEK293 cells (90%) were transiently transfected with 2 µg of a 3xPPRE-TK-Luc luciferase reporter construct (a kind gift from Prof. Walter Wahli at the Université de Lausanne), 500 ng PPARγ also from Prof. Walter Wahli) and 500 ng retinoid X receptor (a kind donation from Prof. David Mangelsdorf at the Southwestern Medical Center, Dallas, Texas), using polyethyleneimine (Poly-sciences Inc.). 6 h after transfection, cells were treated for 24 h with the PPARγ agonist rosiglitazone or cannabidiol. Cells were...
harvested and assayed for luciferase activity (as a measure of reporter gene transcription) using a Luciferase Reporter Assay (Promega; according to the manufacturer’s instructions). Protein content of the cells was determined using the BioRad protein assay according to the manufacturer’s instructions.

2.4. PPARγ binding

Binding to the PPARγ-ligand binding domain was measured using the PolarScreen™ PPARγ competitor assay kit (Invitrogen). Briefly, the competitors were diluted in reaction buffer in a black 384 shallow well plate (NUNC). The purified PPARγ-ligand binding domain and fluorescent PPARγ ligand (Fluormone™ PPAR Green) were then added. Samples were incubated at room temperature for 2 h and fluorescence polarisation obtained by reading the plate using a PerkinElmer Envision 2102 Multi-label Reader with an excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.5. Adipocyte differentiation assay

3T3L1 fibroblasts were grown until confluent and then treated with 1 µM dexamethasone and 5 µg/µl insulin, to induce differentiation, for 2 days at 10% CO₂. Subsequently, cells were kept in maintenance medium consisting of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum supplemented with insulin (5 µg/µl) at 10% CO₂. During this time, cells were treated for 7 days with either increasing concentrations of cannabidiol or rosiglitazone (10 µM). Cells were stained with Oil Red O, to determine lipid accumulation (Mueller et al., 2002; O’Sullivan et al., 2005c), and counterstained with Cresyl Blue.

2.6. Statistical analysis

In each protocol, the number of animals in each group is represented by n, and values are expressed as mean ± S.E.M. The difference between cannabidiol-treated and vehicle-treated vessels under each experimental protocol was analysed by paired Student’s t-test (Fig. 1A). Other data, including net vasorelaxant values, were compared, as appropriate, by analysis of variance (ANOVA) with statistical significance between manipulations and controls determined by Dunnett’s post-hoc test (Figs. 2, 3, 4A, B, and 5A, Tables 1 and 2).

The concentration of vasorelaxant giving half-maximal responses (EC₅₀) and maximal responses (Rₘₐₓ) were obtained from concentration–response curves fitted to a sigmoidal logistic equation using the GraphPad Prism package [\( Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{LogEC₅₀} - \text{X}) \times \text{Hillslope}}) \)], where X is a logarithm of agonist concentration and Y is the response that starts from the Bottom and goes to the Top in a sigmoid shape. Maximal responses (Rₘₐₓ) and pEC₅₀ (negative logarithm of the EC₅₀) values are expressed as mean ± S.E.M. (Fig. 4C).

![Fig. 1. A, The mean vasorelaxant response of cannabidiol vs vehicle (EtOH) over 2 h in a pre-constricted rat aorta. B, The net vasorelaxant effect of cannabidiol (that is, the vasorelaxant effect of cannabidiol minus the vasorelaxant effect of vehicle in the same artery). Data are given as means with error bars representing S.E.M. (n = 8–15).](image1)

![Fig. 2. The effects of PTX treatment (A, 200 ng/ml, 2 h), the cannabinoid CB₁ receptor antagonist AM251 (1 µM) and the cannabinoid CB₂ receptor antagonist AM630 (8, 1 µM), and capsaicin pre-treatment (C, 10 µM, 1 h) on the net vasorelaxation to cannabidiol. Data are given as means with error bars representing S.E.M.](image2)
2.7. Drugs

All drugs were supplied by Sigma Chemical Co. (Poole, UK) except where stated. GW9662 (2-Chloro-5-nitro-N-phenylbenzamide), AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and AM630 (6-Iodo-2-methyl-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methane) were obtained from Tocris (UK). Cannabidiol was generously supplied by GW Pharmaceuticals. Acetylcholine, methoxamine, U46619 (9,11-dideoxy-9alpha,11alpha-methanoepoxy-prosta-5E,13E-dien-1-0ic acid) and PTX were dissolved in distilled water. L-NAME was dissolved in the Krebs–Henseleit solution. Cannabidiol, capsaicin and verapamil were dissolved in ethanol to a stock concentration of 10 mM with further dilutions made in distilled water. GW9662, AM251 and AM630 were dissolved in DMSO to 10 mM, with further dilutions made in distilled water.

3. Results

3.1. Time-dependent effects of cannabidiol in the aorta

At 100 nM, the vascular effect of cannabidiol was not significantly different to vehicle (Fig. 1A). At 1 µM, the effect of cannabidiol was only significantly different from vehicle at 105 and 120 min (2 h, cannabidiol 31.3 ± 6.9% relaxation, n = 9, P < 0.05, Fig. 1A). At 5 µM, the vascular effect of cannabidiol was significantly different from vehicle at all time-points (2 h, cannabidiol 51.9 ± 5.4% relaxation, n = 8, P < 0.001, Fig. 1A). Similarly, 10 µM cannabidiol caused significant time-dependent relaxation of the rat aorta compared to vehicle control at all time-points (2 h, vehicle 18.0 ± 2.5% vs cannabidiol 70.2 ± 3.4% relaxation, n = 15, P < 0.0001, Fig. 1A). After 2 h, the net relaxation (the vasorelaxant effect of cannabidiol minus the vasorelaxant effects of vehicle/time in adjacent segments of aorta) was; 100 nM, 3.6 ± 2.6; 1 µM, 18.1 ± 7.3; 5 µM, 37.1 ± 5.4; 10 µM, 52.2 ± 3.1% relaxation (Fig. 1B). The pEC50 for the time-dependent vasorelaxant effects of cannabidiol is −5.3 ± 0.4 (around 5 µM). Cannabidiol had no effect on vascular tone in aortae not contracted pharmacologically (2 h, vehicle −0.02 ± 0.01 g cf. cannabidiol −0.03 ± 0.01 g, n = 7).

Pre-treating arteries with PTX (200 ng/ml, 2 h) had no effect on the vascular response to cannabidiol (Table 1, Fig. 2A). Neither the cannabinoid CB1 receptor antagonist AM251 (1 µM) nor the cannabinoid CB2 receptor antagonist AM630 (1 µM) had any

Fig. 3. The effects of the PPAR antagonist (GW9662, 1 µM, A), removing the endothelium and inhibiting nitric oxide synthase (L-NAME, 300 µM, B), and inhibition of SOD activity (DETCA, 3 mM, C) on the net vasorelaxation to cannabidiol. Data are given as means with error bars representing S.E.M. *P < 0.05, **P < 0.01, compared to control (ANOVA).

Fig. 4. The vasorelaxant effects of cannabidiol in aortae contracted with (A) a high-K+ Krebs solution and (B) U46619 in a Ca2+-free Krebs–Henseleit buffer. C, The effects of increasing concentrations of cannabidiol (1–30 µM, 10 min) on the contractile response to calcium in a Ca2+-free, high potassium buffer. Data are given as means with error bars representing S.E.M. *P < 0.05, **P < 0.01, compared to control (ANOVA).
significant effect on the time-dependent vascular responses to cannabidiol (Table 1, Fig. 2B). Similarly, pre-treating arteries with the TRPV1 agonist capsaicin (10 µM, 1 h) had no effect on the time-dependent vascular response to cannabidiol (Table 1, Fig. 2C).

In the presence of the PPARγ receptor antagonist GW9662 (1 µM), the net vasorelaxant effect of cannabidiol (the vascular effect of cannabidiol in the presence of GW9662 minus the vasorelaxant effect of vehicle in the presence of GW9662) was significantly reduced from 45 min onwards (Fig. 3A, Table 1). The vasorelaxant effects of cannabidiol were not different in endothelium-denuded and control aortae (Table 1, Fig. 3B). Similarly, in the presence of the nitric oxide synthase inhibitor, L-NAME (300 µM), the net vasorelaxant effect of cannabidiol was not different to that observed in control conditions (Table 1, Fig. 3B). In the presence of the SOD inhibitor, DETCA, the vasorelaxant effect of cannabidiol was signifi-

3.2. Transactivation assays

To determine whether cannabidiol stimulates PPARγ, transactivation assays were performed in HEK293 cells transiently over-expressing PPARγ and retinoid X receptor α in combination with a luciferase reporter gene (3xPPRE TK luc). A, Compared with untreated cells, a significant increase in luciferase activity was seen after treatment with rosiglitazone (10 µM), and with 10 µM and 20 µM cannabidiol. B, Rosiglitazone, ajulemic acid and cannabidiol displaced the fluorescent Fluormone™ PPAR Green ligand from the PPAR-LBD/Fluormone™ PPAR Green complex, resulting in a reduction of polarisation value. Data are given as means and with error bars representing S.E.M. ** denotes a significant difference (P<0.01) compared to vehicle control (ANOVA).

3.3. PPARγ binding

To determine whether cannabidiol directly binds to the PPARγ ligand binding domain, a fluorescence polarisation assay was performed. As shown in Fig. 5B, binding of the PPARγ fluorescent ligand was inhibited in a concentration-dependent manner by rosiglitazone (IC50~35 nM), ajulemic acid (IC50~600 nM) and cannabidiol (IC50~5 µM).

3.4. Induction of adipocyte differentiation

3T3L1 cells were cultured until confluent and then treated for 8 days with either cannabidiol or rosiglitazone. Cells were fixed and stained with Oil Red O to identify fat droplets, the presence of which indicates differentiation of fibroblasts into adipocytes. As shown in Fig. 6A, untreated cells showed some signs of differentiation, but the majority of cells retained their spindle shape with little Oil Red O staining. Rosiglitazone (Fig. 6B) induced differentiation of 3T3 L1 cells to adipocytes, as evidenced by large amounts of Oil Red O staining indicating fat droplet accumulation within the cytoplasm. In the presence or absence of the PPARγ antagonist GW9662 (Fig. 4B). The potency and maximal contractile response to the re-introduction of calcium in calcium-free, high potassium Krebs–Henseleit solution was significantly reduced by cannabidiol in a concentration-dependent manner (1 µM to 30 µM; see Table 2, Fig. 4C). The maximal vasorelaxant response to 10 µM cannabidiol was similar to that of the calcium channel blocker, verapamil (cannabidiol 70±3% relaxation, n = 15 vs 10 µM verapamil 64±9% relaxation).

Table 1

<table>
<thead>
<tr>
<th>Mechanisms underlying the vasorelaxant effect of cannabidiol.</th>
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<tbody>
<tr>
<td>1 h % relaxation</td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>Cannabidiol</td>
</tr>
<tr>
<td>&amp; GW9662</td>
</tr>
<tr>
<td>Endothelium denuded</td>
</tr>
<tr>
<td>&amp; L-NAME (300 µM)</td>
</tr>
<tr>
<td>&amp; DETCA (3 mM)</td>
</tr>
<tr>
<td>&amp; AM251 (1 µM)</td>
</tr>
<tr>
<td>&amp; AM630 (1 µM)</td>
</tr>
<tr>
<td>&amp; PTK (200 ng/ml, 2 h)</td>
</tr>
<tr>
<td>&amp; Capsaicin (10 µM, 1 h)</td>
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<tr>
<td>High K+ contracted</td>
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<td>U46619 contracted (Ca2+ free)</td>
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<tr>
<td>U46619 contracted (Ca2+ free) &amp; GW9662 (1 µM)</td>
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</table>

Residual relaxation (the vasorelaxant effect of cannabidiol minus the vasorelaxant effect of vehicle in an adjacent segment of artery under the same experimental conditions) to cannabidiol after 1 or 2 h under various experimental conditions.

ab (P<0.05, ANOVA) denotes a significant difference between vasorelaxation to cannabidiol under control conditions, and those under experimental conditions (P<0.01).
presence of cannabidiol, concentration-dependent fat droplet accumulation was apparent at all concentrations tested (Fig. 6C–F).

4. Discussion

The aim of the present study was to establish whether the phytocannabinoid, cannabidiol, causes time-dependent vasorelaxation of the rat aorta similar to THC (O’Sullivan et al., 2005c), and whether this is due to PPARγ activation. We have shown, for the first time, that cannabidiol causes a time-dependent vasorelaxant effect that is partially inhibited by a PPARγ antagonist. We have also shown that cannabidiol causes significant activation of the transcriptional activity of PPARγ, binds directly to PPARγ and causes the induction of adipogenesis, a PPARγ ligand property. Together, these data strongly suggest that cannabidiol is a functional PPARγ agonist.

We have previously shown that THC produces time-dependent vasorelaxation of isolated aortae mediated by PPARγ (O’Sullivan et al., 2005c). We have now investigated whether similar responses are produced by cannabidiol, and have shown that cannabidiol (maximum net relaxation 52%), causes a time-dependent progressive vasorelaxant effect similar to that of THC (maximum net relaxation 47%) and rosiglitazone, (maximum net relaxation 70%) (Cunnane et al., 2004; O’Sullivan et al., 2005c). To test whether this response is due to PPARγ activation, some experiments were performed in the presence of the PPARγ antagonist, GW9662, which caused a significant reduction in the vasorelaxant response to cannabidiol, as previously observed for THC and rosiglitazone (O’Sullivan et al., 2005c).

We demonstrated that that the time-dependent effects of THC were abolished in the presence of a SOD inhibitor, DETCA (O’Sullivan et al., 2005c). It was similarly observed in the present study that DETCA inhibited the time-dependent vasorelaxant effects of cannabidiol, suggesting that increased SOD activity promotes vasorelaxation through reductions in reactive oxygen species. This is in agreement with other works showing PPARγ ligands cause the induction of Cu/Zn-SOD (Hwang et al., 2005).

Some of the vasorelaxant effects of cannabinoids are due to activation of other target sites (see Randall et al., 2004 for a review), and we explored whether the vasorelaxant response to cannabidiol might be partially mediated by any of these. We found that neither the cannabinoid CB1 nor cannabinoid CB2 receptor antagonists had any effect on vasorelaxation to cannabidiol. These data are supported by our finding that the vasorelaxant effect of cannabidiol was not affected by the G(γ/o)-protein inactivator PTX, and suggest that the time-dependent vasorelaxant effects of cannabidiol are not mediated through as yet unidentified G(γ/o)-protein-coupled cannabinoid receptors. It is also unlikely that cannabidiol acts through the proposed G-protein-coupled endothelial cannabinoid receptor as the vasorelaxant response to cannabidiol was not sensitive to either removal of the endothelium or PTX treatment, as previously shown for ligands of this receptor (Mo et al., 2004; Begg et al., 2003). Additionally, we previously have demonstrated that sensitivity to the endothelial cannabinoid receptor antagonist, O-1918, only occurs in small resistance arteries of the mesenteric bed and not in conduit arteries like the aorta (O’Sullivan et al., 2004). It is also not likely that the vasorelaxant response to cannabidiol is mediated by the proposed cannabinoid receptor, GPR55, as cannabidiol is not suggested to be an agonist at this receptor (Ryberg et al., 2007). The time-dependent vasorelaxant response to cannabidiol was not inhibited by pre-incubation with capsaicin, to deplete sensory neurotransmitters, making it unlikely that cannabidiol acts via TRPV1 in the aorta.

Some of the vasorelaxant effects of endocannabinoids and THC are brought about by potassium channel activation and calcium channel inhibition (Randall et al., 1997; Ho and Hiley, 2003; O’Sullivan et al., 2005b). The potential involvement of K+ channel activation by cannabidiol was therefore investigated by assessing the vasorelaxant

Table 2
The effects of cannabidiol on the contractile response to calcium.

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>pEC50</th>
<th>Rmax (g increase in tension)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>3.49 ± 0.08</td>
<td>2.49 ± 0.05</td>
<td>11</td>
</tr>
<tr>
<td>1 µM cannabidiol</td>
<td>3.22 ± 0.14</td>
<td>2.10 ± 0.13</td>
<td>8</td>
</tr>
<tr>
<td>10 µM cannabidiol</td>
<td>2.87 ± 0.11</td>
<td>1.86 ± 0.11</td>
<td>11</td>
</tr>
<tr>
<td>30 µM cannabidiol</td>
<td>2.24 ± 0.10</td>
<td>1.38 ± 0.11</td>
<td>6</td>
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* (P<0.05, ANOVA) denotes a significant difference between vehicle and cannabidiol-treated aortae (P<0.01).
response to cannabidiol in vessels pre-contracted with a high potassium buffer to inhibit potassium flux. Under these conditions, a small reduction in the vasorelaxant response to cannabidiol was observed at 45 min, suggesting that activation of potassium channels leading to hyperpolarisation and vascular relaxation is not the primary mechanism by which cannabidiol acts. In further experiments, some aortae were contracted with U46619 in a calcium-free buffer, a condition in which contraction is independent of calcium influx (Williams and Roberts, 2003). In these experiments, the vasorelaxant effect of cannabidiol was considerably reduced, suggesting the mechanism by which cannabidiol acts involves calcium. To investigate this further, we performed concentration–response curves to CaCl₂ in Ca²⁺-free buffers in the absence and presence of cannabidiol, and it was found that cannabidiol significantly inhibited the contractile response to calcium in a concentration-dependent manner. Taken together, these data suggest that the vasorelaxant effect of cannabidiol is mediated by calcium channel inhibition. While the possibility exists that the inhibition of Ca²⁺ channels may be as a consequence of PPAR activation, this is unlikely, as cannabidiol inhibited the contractile response to calcium after only 10 min incubation. Interestingly, other PPAR agonists have been reported to cause non-PPAR-mediated acute relaxation of resistance arteries via blockade of Ca²⁺ channels (Heppner et al., 2005).

To summarise the previously unreported vascular effects of cannabidiol in the rat aorta, we have shown that cannabidiol causes time-dependent vasorelaxation that is neither endothelium–nor NO-dependent, does not involve cannabinoid CB₁, cannabinoid CB₂ receptors or TRPV1 channel activation, but appears to be partly due to inhibition of calcium channels. The time-dependent vasorelaxant effects of cannabidiol were also antagonised by the PPARγ antagonist, GW9662, and by the SOD inhibitor, DETCA.

Increasing evidence has indicated that cannabinoids are capable of binding to, activating and causing PPAR-mediated responses (see O'Sullivan, 2007). We have shown that the major active ingredient of cannabis, THC, activates PPARγ (O’Sullivan et al., 2005, 2006). To test whether cannabidiol similarly activates PPARγ, a number of experiments were performed. Transactivation assays employed homologous cells transiently over-expressing PPARγ and retinoid X receptor α in combination with a luciferase reporter gene. In these assays, the synthetic PPARγ agonist rosiglitazone significantly stimulated the transcriptional activity of PPARγ. We also found that cannabidiol significantly increased the transcriptional activity of PPARγ at 10 μM and 20 μM. In a competition-binding assay, cannabidiol displaced a fluorescent PPARγ ligand with an IC₅₀ of approximately 5 μM, indicating a much lower affinity than was found for either rosiglitazone or ajulemic acid. As further confirmation that cannabidiol is a PPARγ ligand, we examined the ability of cannabidiol to stimulate fat cell differentiation in 3T3 L1 fibroblasts, a well-known property of PPARγ ligands (Mueller et al., 2002). At all concentrations tested, from 100 nM to 10 μM, cannabidiol was found to stimulate the differentiation of fibroblasts to adipocytes, shown by the presence of fat droplets stained red with Oil Red O. Taken together, these data show for the first time that cannabidiol is a PPARγ ligand capable of binding to and increasing the transcriptional activity of PPARγ. We have also demonstrated PPARγ-mediated effects of cannabidiol both in the vasculature and in adipocytes.

Recent crystallography studies using ajulemic acid suggest that this compound occupies about 30% of the ligand binding cavity of PPARγ and forms polar contacts mainly with the ω-loop, and not the C-terminal helix H12, as has been observed for other ligands (Ambrosio et al., 2007). This may explain why cannabinoids such as anandamide, ajulemic acid and cannabidiol are less potent compared to synthetic ligands. However, it should be noted that, despite the low affinity of cannabidiol in the PPARγ binding assay, similar increases in transcriptional activity and adipogenesis were observed when compared to rosiglitazone, suggesting that cannabidiol has good efficacy at PPARγ.

It is generally recognised that there are several side effects associated with PPARγ ligands, including weight gain, oedema and increased plasma lipoproteins (Gelman et al., 2007). New PPARγ agonists that do not possess these side effects are currently being investigated, and it is suggested that partial or low affinity agonists may be beneficial (Gelman et al., 2007). Cannabidiol may, therefore, prove to have therapeutic utility as a low affinity agonist of PPARγ.

In summary, these data provide strong evidence, for the first time, that cannabidiol is a PPARγ agonist, and suggest a novel means by which the diverse effects of cannabidiol could be brought about. In light of the evidence that PPARγ ligands have beneficial effects in type 2 diabetes, the cardiovascular system and, potentially, in a wide variety of other disorders including cancer, gastro-inflamatory disorders and many skin diseases, we provide a further rationale for the investigation of natural cannabinoids as therapeutic agents.

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References


