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RESEARCH PAPER

In vivo effects of CB_2 receptor-selective cannabinoids on the vasculature of normal and arthritic rat knee joints

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Background and purpose: Cannabinoids (CBs) are known to be vasoactive and to regulate tissue inflammation. The present study examined the *in vivo* vasomotor effects of the CB_2 receptor agonists JWH015 and JWH133 in rat knee joints. The effect of acute and chronic joint inflammation on CB_2 receptor-mediated responses was also tested.
Experimental approach: Blood flow was assessed in rat knee joints by laser Doppler imaging both before and following topical administration of CB_2 receptor agonists. Vasoactivity was measured in normal, acute kaolin/carrageenan inflamed and Freund's complete adjuvant chronically inflamed knees.
Key results: In normal animals, JWH015 and JWH133 caused a concentration-dependent increase in synovial blood flow which in the case of JWH133 was blocked by the selective CB_2 receptor antagonist AM630 as well as the transient receptor potential vanilloid-1 (TRPV1) antagonist SB366791. The vasodilator effect of JWH133 was significantly attenuated in both acute and chronically inflamed knees. Given alone, AM630 had no effect on joint blood flow.
Conclusion and implications: In normal joints, the cannabinomimetic JWH133 causes hyperaemia via a CB_2 and TRPV1 receptor mechanism. During acute and chronic inflammation, however, this vasodilatatory response is significantly attenuated.
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Keywords: arthritis; blood flow; cardiovascular system; cannabinoids; CB_2 receptor; endocannabinoids; knee joint; laser doppler imaging; transient potential vanilloid receptor

Abbreviations: CB, cannabinoid; LDI, laser Doppler perfusion imaging; MAP, mean arterial pressure; PU, perfusion unit; TRPV1, transient receptor potential vanilloid-1

Introduction

Cannabis sativa is the source of at least 60 distinct alkaloids, which make up a group of compounds called cannabinoids. Cannabinoids can be categorized depending on whether they are synthetic, plant-derived (phytocannabinoids) or naturally produced in the body (endocannabinoids). The endocannabinoid system is believed to play an important role in health and disease where it can ameliorate the severity of disorders, such as pain, multiple sclerosis, schizophrenia and emesis (Di Marzo and Petrocellis, 2006; Pertwee, 2006a). Overproduction of endocannabinoids, however, could have a detrimental effect on the body, leading to problems such as obesity, infertility and inflammatory disorders. Thus, strategic control of the endocannabinoid system with selective agonists or antagonists may prove to have therapeutic value in the treatment of various diseases.

Two cannabinoid receptors have been cloned, viz. CB_1 and CB_2 (Matsuda *et al.*, 1990; Munro *et al.*, 1993). These $G_{i/o}$ protein-coupled receptors are distributed throughout the body and are involved in the control of miscellaneous physiological processes, such as pain perception, appetite and vasoregulation. CB_1 receptors are predominantly found on nerve terminals in the central and peripheral nervous systems (Tsou *et al.*, 1998; Pertwee, 1999a; Farquhar-Smith *et al.*, 2000), although they have also been localized in non-neuronal tissues, such as the pituitary gland, spleen and immunocytes (Bouaboula *et al.*, 1993). The primary location of CB_2 receptors is on immunocytes (Pertwee, 1999b, 2006a), but they have also been identified on peripheral nerves (Griffin *et al.*, 1997) and in the central nervous system (Van Sickle *et al.*, 2005). A plethora of cannabinoid ligands have been developed with fairly high selectivity for CB_1 and CB_2 receptors (Pertwee, 1999b, 2006b). Two selective CB_2

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receptor agonists are JWH015 ((2-methyl-1-propyl-1H-indol-3-yl)-1-napthalenylmethanone) and JWH133 ((6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[b,d]pyran). The K_i values for JWH015 are 383 and 13.8 nM for the CB_1 and CB_2 receptors, respectively (Chin *et al*., 1999), while JWH133 has K_i of 677 and 3.4 nM for the CB_1 and CB_2 receptors, respectively (Huffman *et al*., 1999). For antagonists, the aminoalkylindole AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl) methanone) has been shown to be an effective blocker of CB_2 receptors (Hosohata *et al*., 1997).

Cannabinoids are known to exert potent cardiovascular effects *in vivo*. For example, systemic administration of the endocannabinoid anandamide, which acts on CB_1 and CB_2 receptors, causes hypotension and bradycardia (Varga *et al*., 1996; Högestatt and Zygmunt, 2002). Moreover, anandamide has been shown to produce vasodilatation in the brain (Ellis *et al*., 1995), liver (Garcia *et al*., 2001), kidney (Deutsch *et al*., 1997), heart (Wagner *et al*., 2001; Ford *et al*., 2002) and mesentery (Randall *et al*., 1996; White and Hiley, 1998; Jarai *et al*., 1999). A number of studies using more selective cannabinoid analogues have confirmed that CB_1 receptor activation leads to relaxation of vascular smooth muscle and systemic hypotension (for review, see Randall *et al*., 2004). Indeed in the rat knee joint, local administration of the selective CB_1 receptor agonist, arachidonyl-2-chloroethylamide causes an increase in synovial blood flow (Baker and McDougall, 2004). Less is known about the vasomotor effects of CB_2 receptor agonists. Wagner *et al*. (2005) reported that *in vitro* perfusion of rat hearts with JWH133 caused an increase in coronary blood flow that was insensitive to AM630 antagonism. *In vivo* assessment of CB_2 receptor agonists on tissue blood flow have so far not been reported.

Evidence has been emerging in recent years that suggests that cannabinoids are able to bind to and activate the transient receptor potential vanilloid-1 (TRPV1) channel. TRPV1 channels are non-selective cation channels expressed on the axon terminals of a subgroup of primary afferent neurons (Caterina *et al*., 1997; Szallasi, 2002). In joints, activation of TRPV1 channels by local capsaicin treatment leads to inflammatory changes, including increased synovial blood flow (Karimian *et al*., 1995; Varga *et al*., 2005). Interestingly, the CB_1 receptor agonist arachidonyl-2-chloroethylamide has also been shown to act on TRPV1 channels to cause synovial hyperaemia in the rat knee (Baker and McDougall, 2004). Whether CB_2 receptors are similarly coupled to TRPV1 channels has never been explored.

The current study was undertaken to assess the vasomotor effects of the two CB_2 receptor agonists, JWH015 and JWH133, on knee joint blood flow *in vivo*. The role of TRPV1 channels in these vasoregulatory processes was also investigated. Further studies were carried out to examine the effect of joint inflammation on CB_2 receptor-mediated responses using animal models of acute and chronic arthritis.

Methods

All experimental procedures received prior approval from the University of Calgary Animal Care Committee, which is in accordance with the Canadian Council for Animal Care. Adult male Wistar rats (242–495 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada) and housed in pairs at room temperature under a 12 h light–dark cycle, with free access to water and standard rat chow. Animals were randomly assigned to one of three experimental groups: naïve control, acutely inflamed and chronically inflamed.

Induction of joint inflammation

Two distinct models of arthritis were used in the present investigation based on the different phases of inflammation. Thus, the kaolin/carrageenan arthritis model produces an acute inflammatory reaction within 24 h after induction, while adjuvant monoarthritis is more suitable to assess the chronic phase of joint inflammation. Prior to arthritis induction, knee joint diameters were measured using electronic digital callipers (Mitutoyo Instruments, Tokyo, Japan) oriented medio laterally across the joint line between the femoral condyle and the tibial plateau. Diameters were compared before and upon completion of inflammation development, which is at 24 h for the kaolin/carrageenan model and at 1 week for the adjuvant monoarthritis model.

In the acutely inflamed group of rats, inflammation was induced in the right knee joint by kaolin and carrageenan. Under isoflurane anaesthesia (2–5% isoflurane; 100% O_2 at 1 l min^{-1}), 0.2 ml of 2% kaolin was injected through a 27-gauge needle into the posterior and anterior synovial cavity, followed by repeated limb extensions and flexions for 10 min to ensure adequate dispersion of the suspension within the joint and to cause articular abrasion. Subsequently, 0.2 ml of 2% carrageenan was injected into the joint by the same procedure. These animals were allowed to recover for 24 h prior to blood flow assessment.

For chronic inflammation, rats were anaesthetized with 2–5% isoflurane (100% O_2 at 1 l min^{-1}), and a localized monoarthritis was induced as previously described (Donaldson *et al*., 1993; McDougall *et al*., 1995). The right knee joint was shaved and 0.2 ml of Freund's complete adjuvant (heat-killed *Mycobacterium tuberculosis*, 1 mg ml^{-1}) was injected through a 27-gauge needle into the knee joint with 0.1 ml being introduced into the posterior compartment of the joint and 0.1 ml being injected anteriorly. Animals were allowed to recover for 1 week prior to the blood flow experiments. Sham-injected animals were not tested as an intra-articular injection of sterile saline has been shown to have no effect on synovial blood flow or vasoreactivity (Karimian *et al*., 1995).

Surgical preparation of animals

On the day of experimentation, animals were deeply anaesthetized with urethane (25% stock solution, 2 g kg^{-1}, i.p.). Depth of anaesthesia was confirmed by a lack of a pinch withdrawal reflex applied to the hindpaw as well as an absence of a corneal blink reflex. A longitudinal incision was made in the neck and the trachea was isolated and cannulated to permit unobstructed breathing. The right carotid artery was then isolated and cannulated with

heparinized saline (100 U ml^{-1})-filled polyethylene tubing (Portex Fine Bore Tubing, 0.5 mm ID, 1.00 mm OD; SIMS Portex Ltd., Kent, UK). The carotid cannula was attached to a pressure transducer (1050 Pressure Transducer; Stoelting Co., Wood Dale, IL, USA) and mean arterial pressure (MAP) was recorded continuously throughout the experiment via a blood pressure monitor (Pressure Monitor BP-1; World Precision Instruments, Sarasota, FL, USA). The animal was then placed in dorsal recumbency on an electric-heating blanket (TR-100; Fine Science Tools Inc., Vancouver, British Columbia, Canada) and the internal body temperature was maintained at 37 °C as measured by a rectally inserted electronic thermometer. An ellipse of skin covering the anteromedial region of the right knee joint was removed and the underlying tissue was kept moist by regular superfusion of 37 °C physiological saline (0.9% NaCl).

Measurement of knee joint blood flow

Knee joint blood flow was measured by laser Doppler perfusion imaging (LDI) as previously described (Karimian *et al*., 1995; McDougall *et al*., 1995). The technique involved placing a laser Doppler perfusion imager (MoorLDI V.2; Moor Instruments Ltd., Devon, UK) 30 cm above the exposed rat knee and angling the scanner head to avoid reflectance artefact. A low-power (2 mW) red He–Ne laser ($\lambda = 633$ nm) was directed onto the surface of the knee joint and by means of a motor-controlled mirror the laser beam scanned in a raster pattern across the joint capsule. Careful placement of opaque black cloth at the margins of the knee ensured that only the joint capsule was scanned. The image resolution was set at 42 pixels × 55 pixels with a scan speed of 4 ms pixel^{-1}. At each pixel point, photons in the incident laser beam underwent Doppler shift by virtue of erythrocytes flowing in the tissue. These Doppler-shifted photons were captured by a photodetector in the scanner head and a perfusion value was calculated based on the velocity and concentration of erythrocytes circulating in the microvasculature. A two-dimensional colour-coded image of tissue perfusion was then constructed and stored for later offline analysis.

To achieve a more accurate appreciation of the time course for cannabinoid activity, the imager was set to the single-point measurement mode to maximize the temporal resolution of the instrument. In these experiments, the laser beam remained stationary at a discrete locus on the surface of the joint capsule, and blood flow measurements were continuously acquired. The precise positioning of the laser beam was randomly chosen, but was usually placed in the vicinity of an observable microvascular network. A steady blood flow level was measured for about 1 min before a cannabinoid agonist was topically applied to the knee joint. Blood flow was then measured continually over the succeeding 5 min. In subsequent scanning experiments, the timeline for image acquisition was designed so as to coincide with the maximal vasomotor effect of the test agents.

Experimental protocol

All test reagents were placed in a water bath and gently warmed to 37 °C. A control LDI scan of the knee was taken and then the test drug was topically applied to the surface of the joint capsule as a 0.1 ml bolus. The CB_2 receptor agonists tested in this study were JWH015 (10^{-14}–10^{-11} mol) and JWH133 (10^{-14}–10^{-12} mol). Blood flow scans of the joint were then taken at 0, 1.5, 2, 3 and 5 min following drug application. A cumulative concentration–response curve to each agonist was generated in separate groups of animals and sequential doses of agonist were applied to the joint following each 5-min scan. In other experiments, each dose of the CB_2 receptor agonist was co-administered with the selective CB_2 receptor antagonist AM630 (10^{-8} mol; 0.1 ml bolus topical). The concentration of AM630 was based on previous successful antagonism in the rat cardiovascular system (Ford *et al*., 2002). To determine whether the transient potential vanilloid receptor-1 (TRPV1) is involved in CB_2 receptor-mediated vasoregulation, additional experiments were undertaken in which animals were pretreated with the TRPV1 receptor antagonist SB366791. At 20 min following an intraperitoneal injection of SB366791 (500 μg kg^{-1}), concentration–response effects of JWH133 were assessed. This treatment regimen with SB366791 has previously been shown to successfully block TRPV1 receptors in the rat knee joint (Varga *et al*., 2005). It should be pointed out that JWH015 proved to be a weak, non-selective agonist for the CB_2 receptor and as such was neither used in the SB366791 experiments nor tested in the inflammatory models.

At the conclusion of the experiment, the animal was killed by anaesthetic overdose (pentobarbital sodium, 240 mg intracardiac) and a final scan of the joint was performed. This dead scan constituted a 'biological zero' measurement, which corresponded to tissue optical noise and residual Brownian motion. This 'biological zero', which was typically about 5–10% of the basal perfusion level, was subtracted from all image perfusion values prior to data processing.

Image analysis and statistics

Knee joint LDI scans were analysed by proprietary perfusion image-processing software (Moor Instruments Ltd.). For each image, an average blood flow value for the entire anteromedial knee joint capsule was calculated and expressed in arbitrary perfusion units (PUs). Blood flow changes in response to drug administration were expressed as percentage change in perfusion between control and test scans. The maximal vasomotor effect (E_{max}) and the EC_{50} were calculated from a sigmoidal nonlinear regression curve fit. All data sets conformed to a Gaussian distribution and as such were tested with parametric statistical analyses using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). ANOVA was used to assess significant differences in blood flows within (one-way ANOVA) and between (two-way ANOVA) different animal groups. A Bonferroni *post hoc* test was used to determine whether groups of data were significantly different at a specific concentration of cannabinoid. A significance level of $P < 0.05$ was used for each test and all reported values represent means ± s.e.m. for '*n*' observations.

Drugs and reagents

JWH015, JWH133 and AM630 were all purchased from Tocris Bioscience, Ellisville, MO, USA; SB366791, Freund's complete

adjuvant, λ-carrageenan, kaolin, urethane and heparin were all obtained from Sigma-Aldrich Ltd., Oakville, Ontario, Canada. SB366791 and all cannabinoid agonists and antagonists were dissolved in dimethyl sulphoxide and cremophor, diluted to their working concentrations, aliquoted and stored at either 4 °C or −20 °C as required. The final concentration of vehicle was maintained at 2% dimethyl sulphoxide and 1% cremophor for each concentration. This vehicle formulation has previously been found to have no significant effect on joint blood flow (Baker and McDougall, 2004).

Results

CB_2 receptor effects in normal knee joints

LDI-determined basal blood flow in normal rat knees was 421 ± 26.8 PU ($n = 52$). Application of the CB_2 receptor agonists JWH015 and JWH133 onto the surface of normal rat knees caused a marked but transient increase in synovial blood flow. Continuous measurement of joint blood flow at a single discrete location indicated that vasodilatation was rapid in onset in response to CB_2 receptor activation, with maximal blood flow being reached approximately 1.5 min after drug application (Figure 1). Synovial blood flow subsequently returned to control levels at around the 4-min time point. Concentration–response curves were constructed for JWH015 and JWH133 and both agonists showed a concentration-dependent effect (Figure 2). The calculated E_{max} for JWH015 was 18.5 ± 3.2% with an EC_{50} of 1.9×10^{-14} mol. JWH133 on the other hand had an E_{max} of 36.4 ± 5.3% and an EC_{50} of 7.9×10^{-14} mol. The selective CB_2 receptor antagonist AM630 had no effect on JWH015-mediated vasodilatation ($P = 0.81$, two-way ANOVA; $n = 9$–10; Figure 2a). Conversely, co-administration of AM630 with JWH133 significantly inhibited the vasodilatatory effect of the cannabinoid ($P < 0.0001$, two-way ANOVA; $n = 11$–38; Figure 2b).

Administration of 10^{-12} mol JWH133 without previous application of the lower concentrations of the cannabinoid still elicited around a 40% increase in joint blood flow (data not shown), indicating that tachyphylaxis was not occurring in these experiments.

Since JWH015 only produced a weak, non-selective effect on synovial blood flow, the subsequent experiments were only carried out with the more efficacious JWH133.

Role of TRPV1 in CB_2 receptor-mediated vasodilatation

To determine if the TRPV1 ion channel is involved in the activity shown by JWH133, animals were treated with the TRPV1 antagonist SB366791. As shown in Figure 3, the vasodilator effect of JWH133 was blocked in SB366791-treated animals. A two-way ANOVA confirmed that the JWH133 concentration–response curves in SB366791-treated vs non-treated animals were statistically different from each other ($P < 0.0001$; $n = 12$–38).

Joint oedema following inflammation

Comparison of joint diameters before and after inflammation induction confirmed oedema formation in the animal models of arthritis. In kaolin/carrageenan acutely inflamed knees, joint diameter increased from 9.0 ± 0.07 to 15.1 ± 0.44 mm, while chronically inflamed adjuvant monoarthritic knees showed an increase in knee diameter from 9.0 ± 0.13 to 12.2 ± 0.42 mm. Both increases were found to be statistically significant ($P < 0.0001$, paired Student's *t*-test; $n = 10$–13 knees).

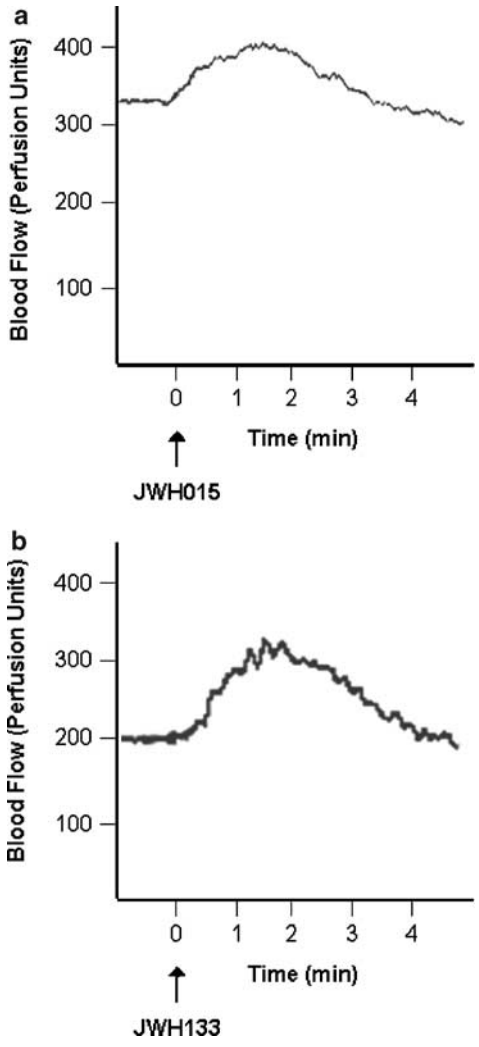

Figure 1 Single-point measurements of knee joint blood flow at a discrete locus on the joint capsule showing typical responses to topical application of the CB_2 receptor agonists JWH015 (**a**) and JWH133 (**b**). Blood flow gradually rose, with maximal vasodilatation occurring 1.5 min after drug administration. Joint blood flow subsequently returned to control levels by about 4 min.

Assessment of JWH133 in inflamed knee joints

Basal blood flow to acutely inflamed knees was 566 ± 65 PU ($n = 10$). Administration of JWH133 to acutely inflamed rat knee joints failed to elicit any significant change in synovial blood flow (Figure 4). A two-factor ANOVA confirmed that the vasodilator effect of JWH133 seen in normal joints was abrogated in the acute inflammatory model ($P < 0.0001$, two-way ANOVA; $n = 8$–12).

In chronically inflamed joints, the hyperaemic effect of JWH133 was also significantly attenuated compared to

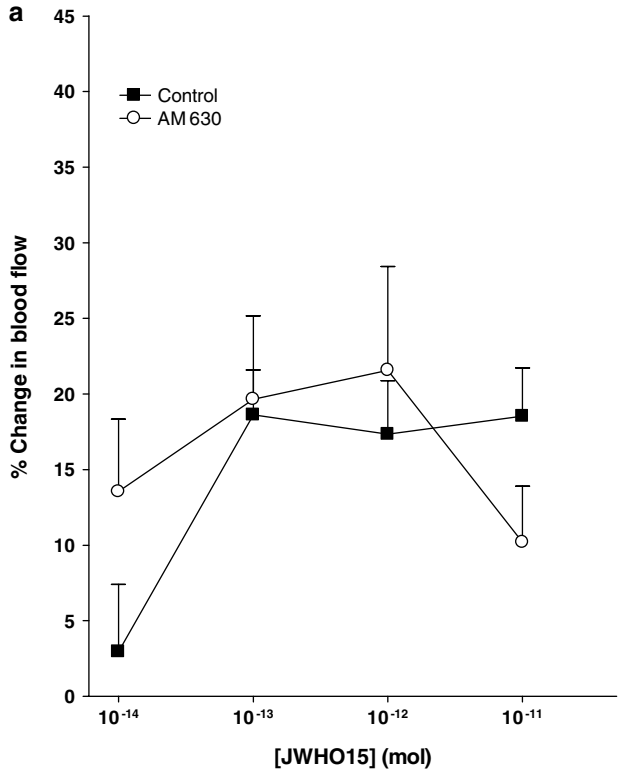

Figure 2 Concentration–response effects of JWH015 (**a**) and JWH133 (**b**) on synovial blood flow. The selective CB_2 receptor antagonist AM630 (10^{-8} mol) was unable to block the vasodilator effect of JWH015, but significantly attenuated the hyperaemic response of JWH133 (***$P<0.001$, **$P<0.01$ vs control; two-way ANOVA followed by Bonferroni *post hoc* test; $n=11$–38). Data are presented as mean percent change in perfusion ± s.e.m.

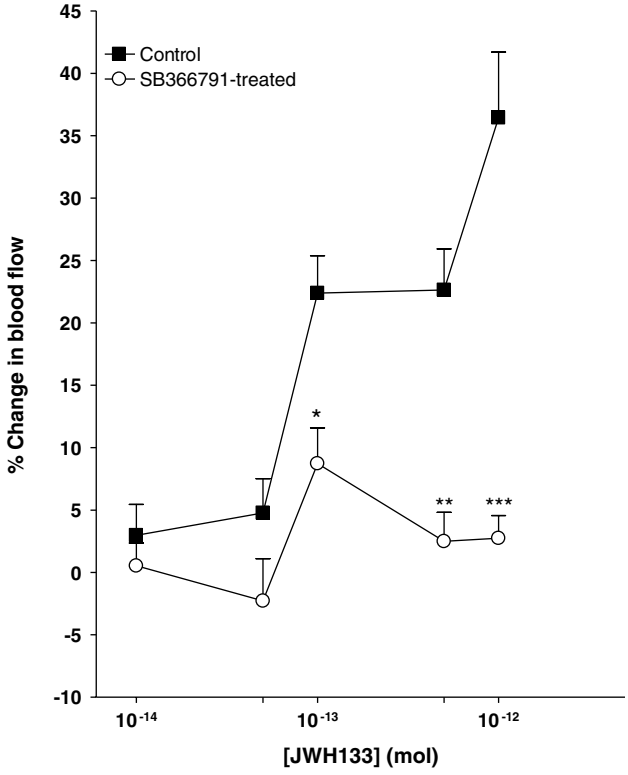

Figure 3 Vasodilator effect of JWH133 is attenuated in animals pretreated with the TRPV1 antagonist SB366791 ($500\,\mu g\,kg^{-1}$). ***$P<0.001$, **$P<0.01$, *$P<0.05$ two-way ANOVA followed by Bonferroni *post hoc* test; $n=12$–38. Data are shown as means ± s.e.m.

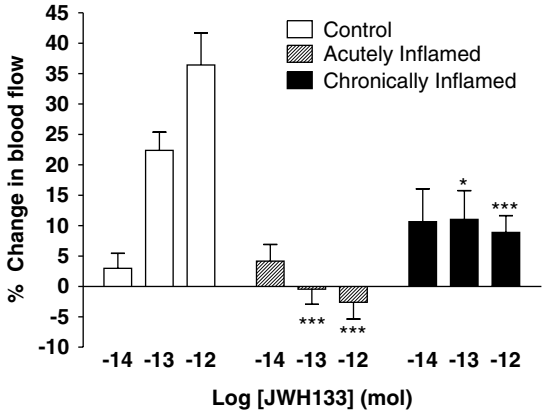

Figure 4 Effect of joint inflammation on JWH133-mediated vasodilatation. The hyperaemic effect of JWH133 was significantly reduced in both acute and chronically inflamed knees (***$P<0.001$, *$P<0.05$ two-way ANOVA followed by Bonferroni *post hoc* test; $n=8$–38). Data are presented as means ± s.e.m.

normal control knees ($P<0.0001$, two-way ANOVA; Figure 4). Basal blood flow to adjuvant monoarthritic knees was 513 ± 47.5 PU ($n=10$).

Effect of AM630 on normal, acutely and chronically inflamed knees

Topical administration of the selective CB_2 receptor antagonist AM630 (10^{-8} mol) had no effect on synovial blood flow

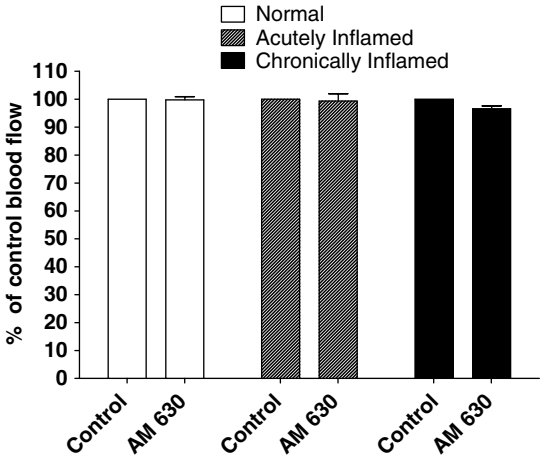

Figure 5 Effect of topical application of the selective CB_2 antagonist AM630 (10^{-8} mol) on knee joint blood flow. AM630 had no effect on joint blood flow in normal, acutely inflamed or chronically inflamed knees. Data are mean % of control values ± s.e.m.

in normal ($P=0.29$), acutely inflamed knees ($P=0.81$) or chronically inflamed ($P=0.05$) rat knee joints (Figure 5). Thus, AM630 does not appear to be acting as an inverse or partial agonist to increase blood flow in this model system.

Blood pressure effects

As shown in Table 1, local administration of all of the test compounds had no significant effect on MAP. Thus, all reported vasomotor changes are a direct effect of the drugs on articular blood vessels and are not a secondary consequence of systemic changes in blood pressure.

Table 1 MAP taken before (control) and at 1.5 min following topical application of the CB2 receptor agonists JWH015 and JWH133, and the CB2 receptor antagonist AM630

Drugs	*MAP (mm Hg)*	n *values*
JWH015 (mol)		
Control	77 ± 5	10
10^{-14}	75 ± 4	10
Control	75 ± 5	10
10^{-13}	73 ± 4	10
Control	74 ± 4	10
10^{-12}	73 ± 3	10
Control	71 ± 3	10
10^{-11}	69 ± 2	10
JWH133 (mol)		
Control	89 ± 3	41
10^{-14}	87 ± 4	38
Control	90 ± 4	38
5×10^{-14}	88 ± 5	28
Control	88 ± 5	28
10^{-13}	82 ± 5	23
Control	82 ± 5	24
5×10^{-13}	75 ± 6	11
Control	85 ± 5	28
10^{-12}	89 ± 6	19
AM630 (mol)		
Control	94 ± 5	17
10^{-8}	93 ± 6	17
SB366971 ($\mu g\,kg^{-1}$)		
Control	94 ± 9	8
500	93 ± 9	10

The effect of the TRPV1 antagonist SB366791 on MAP is also shown (values are expressed as means ± s.e.m.).
Abbreviation: MAP, mean arterial pressure.

Discussion

Cannabinoids acting via the CB_2 receptor are known to heavily influence the activity and function of the nervous system; however, the effect of selective CB_2 receptor agonists on the cardiovascular system are less well understood. The present study found that the synthetic CB_2 receptor agonists JWH015 and JWH133 produced a significant increase in synovial blood flow when applied locally to the joint. These hyperaemic responses were a direct effect of the drugs on the joint microenvironment and not a secondary systemic reaction since the agonists were restricted to the periphery and had no influence on mean arterial blood pressure (see Table 1). Both agonists exhibited similar potencies; however, JWH133 was found to be more efficacious and showed greater selectivity for the CB_2 receptor than JWH015, whose mild vasodilator effect was not blocked by the selective CB_2 receptor antagonist AM630. The difference in responsiveness of these two drugs is likely related to the comparatively low affinity of JWH015 for the CB_2 receptor (Chin *et al*., 1999; Huffman *et al*., 1999). Thus, JWH133 is the pharmacological tool of choice for *in vivo* vasomotor assessment and, as such, follow-up experiments investigating the mechanism of action of cannabinoids and the effect of joint inflammation on CB_2 receptor responses was confined to this particular agonist.

It should be noted that the pharmacological tools currently available for cannabinoid research all possess some limits to selectivity. For example, JWH015, JWH133 and AM630 all show a mild affinity for the CB_1 receptor. Thus, it is entirely feasible that the vasodilator responses observed in this study are being mediated by CB_1 receptors and not CB_2 receptors as reported. Attempts to block CB_1 receptors with an antagonist such as AM251 were not carried out in this study, as AM251 also has a mild affinity for CB_2 receptors. A further consideration is that the JWH compounds may be acting via the putative GPR55 cannabinoid receptor (Johns *et al*., 2007; Ryberg *et al*., 2007) or indeed via other, so far undefined, orphan receptors. Until robust, highly selective drugs become available and all potential cannabinoid receptor subtypes have been identified, the customary caveat with respect to the precise cannabinoid receptors involved in JWH-mediated vasomotor control should be considered.

The site of action of the tested cannabinoids is not readily identifiable in this *in vivo* model. The general viewpoint that CB_2 receptors are localized primarily on immunocytes would indicate that these cells could be involved in the vasomotor effects of JWH015 and JWH133. Indeed, cannabinoids have been shown to activate human leukocytes, leading to the release of vasodilating agents, such as nitric oxide and

pro-inflammatory chemokines (Stefano *et al*., 1996; Sugiura *et al*., 2004). This, however, would not account for the increased blood flow observed in normal joints that do not contain an appreciable number of immunocytes. Alternatively, the cannabinoids could be acting directly on the vascular smooth muscle to cause vasorelaxation. The endocannabinoid anandamide produces vasodilatation by opening large-conductance calcium-activated potassium channels (Grainger and Boachie-Ansah, 2001; White *et al*., 2001; Wagner *et al*., 2005), which causes hyperpolarization of smooth muscle cells and vascular relaxation. The vascular endothelium has also been implicated in the vasodilatatory effect of cannabinoids. A number of *in vitro* studies have shown that cannabinoids can act either as an endothelially derived hyperpolarizing factor or by stimulating the endothelium to cause the secondary release of vasodilator chemicals, such as nitric oxide (Randall *et al*., 1996; Deutsch *et al*., 1997; Stefano *et al*., 2000). A final putative mechanism by which cannabinoids alter tissue blood flow is via their modulatory effects on sensory and sympathetic neurotransmission. Cannabinoids are able to inhibit noradrenaline release from postganglionic sympathetic efferents (Ishac *et al*., 1996; Varga *et al*., 1996; Niederhoffer and Szabo, 1999), thereby attenuating sympathetic vascular tone and leading to hyperaemia. Articular blood vessels are also innervated by sensory nerves containing vasoactive neurotransmitters, such as substance P, vasoactive intestinal peptide and calcitonin gene-related peptide (Bjurholm *et al*., 1990; Abramovici *et al*., 1991; McDougall *et al*., 1997), all of which have been shown to increase synovial blood flow (Lam and Ferrell, 1993; McDougall *et al*., 1995, 1999; McDougall and Barin, 2005). These neuropeptides are released from vasosensory nerves in response to activation of the capsaicin-sensitive TRPV1 channel. Interestingly, anandamide and its synthetic analogues are thought to act on TRPV1 channels to cause the secondary release of pro-inflammatory neurotransmitters, which in turn cause vasodilatation of neighbouring blood vessels (Zygmunt *et al*., 1999; Ralevic *et al*., 2000; Smith and McQueen, 2001; Baker and McDougall, 2004). In the present study, blockade of TRPV1 channels with SB366791 significantly attenuated the vasodilator effect of JWH133. This is the first reported evidence showing that TRPV1 channels are essential for CB_2 receptor functional activity. Similar observations have been described for CB_1 receptor-mediated vasomotor control where the hyperaemic effect of the CB_1 receptor agonist arachidonyl-2-chloroethylamide was attenuated following TRPV1 channel blockade (Baker and McDougall, 2004). It appears, therefore, that both CB_1 and CB_2 receptors are somehow functionally coupled to TRPV1 channels to orchestrate vasoregulation in the knee joint. Whether JWH133 binds directly to TRPV1 receptors to cause secondary release of pro-inflammatory neuropeptides or whether some intracellular pathway exists to allow cross-talk between TRPV1 and CB_2 receptors remains to be resolved. While any combination of the mechanisms discussed above may be responsible for the vasodilator effect of the JWH compounds described here, other pathways cannot be discounted. Indeed, such is the complex nature of cannabinoid biology that several studies report contradictory evidence as to the mechanism of action of these eicosanoids on the vasculature (for reviews, see Mendizabal and Adler-Graschinsky, 2003; Randall *et al*., 2004). Future studies using highly selective cannabinoid agonists in different vascular beds will hopefully reveal the precise mechanism by which CB_2 receptor activation leads to vascular smooth muscle relaxation.

Changes in JWH133-mediated vasodilatation in acute and chronically inflamed knees

When applied topically to acutely inflamed knee joints, JWH133 failed to elicit any significant change in articular blood flow across the concentration range tested. Similarly in chronically inflamed knees, the vasodilator effect of the cannabinoid was conspicuously attenuated compared to normal control joints. One possible explanation for this inability of JWH133 to elicit a hyperaemic response in arthritic knees could be that the synovial microvasculature is already maximally vasodilated such that supplementary smooth muscle relaxation is unattainable in these models. Studies testing other vasodilators in these same inflammatory models, however, have successfully demonstrated that arthritic joint blood vessels possess a vasodilator reserve, indicating that vascular smooth muscle relaxation is still possible in these joints (McDougall and Barin, 2005; Zhang and McDougall, 2006). An alternative reason for the lack of a vasodilatatory response to JWH133 in arthritic knees is that there could be an alteration in articular CB_2 receptor expression and/or sensitivity in inflamed joints; however, this possibility requires further experimental assessment. CB_2 receptor expression on macrophages, microglia, dendritic cells and splenocytes is known to be downregulated following exposure to an inflammatory stimulus (Lee *et al*., 2001; Carlisle *et al*., 2002; Matias *et al*., 2002). Inflammation can cause overactivity of the endocannabinoid system, leading to increased release of anandamide and/or 2-arachidonoylglycerol (Varga *et al*., 1998; D'Argenio *et al*., 2006; Oka *et al*., 2006), and prolonged exposure of cannabinoid receptors to endogenous and synthetic cannabinoid ligands can cause internalization of these $G_{i/o}$ protein-coupled receptors (Fan *et al*., 1996; Rubino *et al*., 2000; Shoemaker *et al*., 2005). In other organ systems, such as the gut, however, there is evidence of cannabinoid receptor upregulation despite elevated endocannabinoid levels (Izzo *et al*., 2001; Massa *et al*., 2004; Kimball *et al*., 2006). It appears, therefore, that the regulation of cannabinoid receptor expression during inflammation depends upon the tissue involved as well as the nature of the inflammatory stimulus. Interestingly, fatty acid amide hydrolase, the enzyme responsible for the degradation of endocannabinoids, is more active in inflamed gut compared to control animals (Izzo *et al*., 2001). This continuous turnover of endocannabinoids during intestinal inflammation implies that endocannabinoids cannot accumulate in the tissue and therefore cannabinoid receptor downregulation is averted. Future examination of fatty acid amide hydrolase activity, cannabinoid receptor expression and endocannabinoid levels in inflamed joints is required to help to explain the altered vasomotor responses to JWH133 demonstrated in the present investigation.

In conclusion, the present investigation clearly demonstrated the functional presence of CB_2 receptors in rat knee joints, whose activation leads to a vasodilator response. Vanilloid TRPV1 channels are essential for the hyperaemic action of JWH133 and further studies are required to determine the molecular and biochemical pathways that link TRPV1 and CB_2 receptors. The attenuation of CB_2 receptor-mediated vasodilatation in acute and chronically inflamed joints suggests an alteration in CB_2 receptor expression or sensitivity following an arthritic insult.

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Conflict of interest

The authors state no conflict of interest.

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