Cannabidiol improves vasorelaxation in Zucker Diabetic fatty rats through cyclooxygenase activation

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Section assignment: Cardiovascular
Running title: Mechanisms of CBD improved vasorelaxation

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Number of text pages: 19
Number of tables: 0
Number of figures: 8
Number of references: 37
Number of words in the Abstract: 239
Number of words in the Introduction: 516
Number of words in the Discussion: 1597

ABBREVIATIONS
ACh = Acetylcholine; CBD = Cannabidiol; COX = Cyclooxygenase; DETCA = Sodium diethylldithiocarbamate trihydrate; EDHF = Endothelium-derived hyperpolarising factor; L-NAME = N\(^{G}\)-nitro-L-arginine methyl ester; NO = Nitric oxide; PEG-catalase = Polyethylene glycol-catalase; SNP = Sodium nitroprusside; THC = \(\Delta^9\)-Tetrahydrocannabinol; TXA\(_2\) = Thromboxane A\(_2\); ZDF rat = Zucker Diabetic Fatty rat.
Abstract

Cannabidiol (CBD) decreases insulitis, inflammation, neuropathic pain and myocardial dysfunction in preclinical models of diabetes. We recently showed that CBD also improves vasorelaxation in the Zucker Diabetic fatty (ZDF) rat, and the objective of the present study was to establish the mechanisms underlying this effect. Femoral arteries from ZDF rats and ZDF lean controls were isolated and mounted on a myograph and incubated with CBD (10 μM) or vehicle for 2h. Subsequent vasorelaxant responses were measured in combination with various interventions. Prostaglandin metabolites were detected using enzyme immunoassay. Direct effects of CBD on cyclooxygenase (COX) enzyme activity were measured by oxygraph assay. CBD enhanced the maximum vasorelaxation to acetylcholine (ACh) in femoral arteries from ZDF lean (P<0.01) and especially ZDF rats (P<0.0001). In ZDF arteries, this enhancement persisted after CB1, CB2 or PPARγ antagonism, but was inhibited by CB2 receptor antagonism. CBD also uncovered a vasorelaxant response to a CB2 agonist not previously observed. The CBD-enhanced ACh response was endothelium-, nitric oxide- and hydrogen peroxide-independent. It was, however, COX1/2- and superoxide dismutase-dependent and CBD enhanced the activity of both purified COX-1 and COX-2. The CBD-enhanced ACh response in the arteries was inhibited by a prostanoid EP4 receptor antagonist. PGE2 metabolite levels were below the limits of detection, but 6-keto PGF1α was decreased after CBD incubation. This data shows CBD exposure enhances the ability of arteries to relax via enhanced production of vasodilator COX 1/2-derived products acting at EP4 receptors.
Introduction

Cannabidiol (CBD) is a naturally occurring molecule found in the plant *Cannabis Sativa*. Unlike the related molecule ∆9-tetrahydrocannabinol (THC), it does not activate cannabinoid 1 (CB₁) receptors in the brain, and is therefore devoid of the psychotropic actions of THC. Indeed, it has been argued that CBD may mitigate the psychoses associated with cannabis abuse (see Schubart et al., 2011 and references therein). A CBD/THC combination (Sativex/Nabiximols) is currently licensed internationally for the treatment of multiple sclerosis, and CBD alone (Epidiolex) was recently approved as an investigational new drug by the Food and Drug Administration in children with medically intractable epilepsy.

The therapeutic potential of CBD has been widely explored in preclinical models of many other disorders ranging from type 1 and type 2 diabetes (see Di Marzo et al., 2011; Horvath et al., 2012 for recent reviews) to prostate cancer (De Petrocellis et al., 2013). With respect to the former, CBD protects the blood-retinal barrier in models of hyperglycaemia (El-Remessy et al., 2006), decreases insulitis and inflammation in non-obese diabetes-prone (NOD) mice (Weiss et al., 2006; Weiss et al., 2008), decreases endothelial cell activation and monocyte adhesion in hyperglycaemia (Rajesh et al., 2007), decreases neuropathic pain in streptozotocin (STZ)-diabetic mice (Toth et al., 2010) and decreases myocardial dysfunction, cardiac fibrosis, oxidative stress and inflammation in a mouse model of type 1 diabetic cardiomyopathy (Rajesh et al., 2010). Indeed, there is much preclinical evidence to suggest that CBD is beneficial throughout the cardiovascular system, as recently reviewed (Stanley et al., 2013).

CBD is far from a selective compound, and produces effects mediated by a number of target sites of action. It has a relatively poor ability to inhibit the binding of the synthetic cannabinoid agonist [3H]CP55940 to CB₁ and cannabinoid 2 (CB₂) receptors, but is possibly a functional antagonist (or inverse agonist) of these receptors (Pertwee, 2008). Other receptor sites implicated in the actions of CBD include the orphan G-protein coupled receptor GPR55, the putative endothelial cannabinoid receptor (CB₇), the transient receptor...
potential vanilloid 1 (TRPV1) receptor, α1-adrenoceptors, μ opioid receptors and serotonin receptor 1 subtype A (5-HT1A) receptors (Pertwee, 2008). CBD also activates and has physiological responses mediated by peroxisome proliferator activated receptor γ (PPARγ) (O’Sullivan et al., 2009; Esposito et al., 2011; De Filippis et al., 2011). Additional mediators of CBD actions include enzymes capable of metabolising endogenously produced cannabinoids (termed endocannabinoids), such as lipoxygenases, cyclooxygenase-2 (COX-2), and fatty acid amide hydrolase (FAAH) (Watanabe et al., 1996; Costa et al., 2004; Massi et al., 2008).

We recently reported that in vitro incubation with CBD at 1 or 10 µM improved vasorelaxation in aortae and femoral arteries of ZDF rats (Stanley & Wheal et al., 2013). The aim of the present study was to determine the mechanisms underlying this effect of CBD in the vasculature of the ZDF rat. We hypothesised that this could involve activation of PPARγ (O’Sullivan et al., 2009) and/or actions mediated by key vascular enzymes such as COX and SOD (O’Sullivan et al., 2006), and represent a facet of the beneficial effects of CBD in diabetes, or indeed other vascular disorders.

MATERIALS AND METHODS

Animals

Male ZDF rats (Charles River USA, n=51) were housed in groups of 2-4 in the University of Nottingham Biomedical Services Unit (BMSU) with a 12h light/dark cycle, at 22±2°C, and access to chow and water ad libitum. Homozygote recessive males (fa/fa) develop obesity, hyperlipidaemia, fasting hyperglycemia and Type 2 diabetes (ZDF rats, n=35). Wildtypes (+/+) and heterozygous (fa/) lean genotypes remain normoglycaemic (ZDF lean control, n=16). At 12-13 weeks old the rats were stunned by a blow to the head and killed by cervical dislocation. Post-mortem blood glucose concentrations were measured using an Accu-Chek Aviva analyser (Roche Diagnostics Ltd., Mannheim, Germany). All procedures were in accordance with the UK Home Office Animal (Scientific Procedures) Act 1986 and
Nottingham University Ethical Review Panel. ZDF rats were heavier than the ZDF lean control rats (ZDF 384 ± 2 g, n=35, lean 305 ± 14g, n=16; p<0.0001, Students’ t-test, mean ± s.d). Blood glucose levels were also higher in ZDF rats than ZDF lean rats (ZDF 22.2 ± 5.1mmol/L, n=35, lean 7.6 ± 0.7mmol/L, n=16; p<0.0001).

Experimental design

Sections of femoral artery (~2 mm, up to 16 segments of femoral artery per rat) were mounted on a myograph at a basal tension of 4.9 mN (Danish Myo Technology, Aarhus, Denmark) in warmed (37°C), gassed (95% O₂/5% CO₂) modified Krebs’-Henseleit buffer (mmol/L:118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 D-glucose, 2 CaCl₂). PowerLab recording systems were used to record changes in tension (ADInstruments, Oxfordshire, UK). Contraction with high K⁺ buffer (mmol/L: 62.5 NaCl, 59.4 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 D-glucose, 2 CaCl₂) was used to demonstrate the viability of the mounted vessels. Then, arteries were incubated for 2h with either 10μmol/L CBD, or vehicle (5μl ethanol in 5 ml, 0.1%). Following this, arteries were contracted with an α₁-adrenoceptor agonist, methoxamine (10-100μmol/L) to reach a stable tone (at least 4.9 mN above basal tone). In some cases, U46619 (TxA₂ mimetic) up to 100 nM was also added to achieve the required level of tone or to help stabilise tone. Cumulative concentration-response curves to acetylcholine (ACh), sodium nitroprusside (SNP) or the CB₂ receptor agonist HU308 (1 nM-100μmol/L) were constructed.

Potential target sites of action were examined using a range of antagonists (or enzyme inhibitors) acting at PPARγ (GW9662, 1μmol/L), CB₁ (AM251, 1μmol/L), CB₂ (AM630, 1μmol/L), the putative CB₆ (O1918, 1μmol/L), and fatty acid amide hydrolase (URB597, 1μmol/L). In some arteries, the intima was rubbed with forceps to remove the endothelium. The role of endothelium-derived hyperpolarisation was investigated by inhibition of nitric oxide synthase with N⁰-nitro-L-arginine methyl ester (L-NAME, 300μmol/L), inhibition of COX (indomethacin, 3μmol/L), and blockade of small and intermediate calcium-
activated potassium channels with apamin (0.5μmol/L) and TRAM-34 (10μmol/L) respectively.

The involvement of COX was investigated by the co-incubation with the non-selective COX inhibitors indomethacin (3μmol/L) or flurbiprofen (10μmol/L), or a COX-2 selective inhibitor, nimesulide (10μmol/L). The DE/EP prostanoid receptor antagonist AH6809 (1μmol/L), EP4 receptor antagonist L161982 (1μmol/L), and the IP (prostacyclin) antagonist CAY10441 (100nmol/L) were used. To establish any role for hydrogen peroxide and superoxides, arteries were co-incubated with polyethylene glycol (PEG)-catalase (300U/ml) to breakdown active endogenous hydrogen peroxide, or sodium diethyldithiocarbamate trihydrate (DETCA, 300μmol/L) to inhibit superoxide dismutase.

Cyclooxygenase activity assays
The method used was that of Meade et al. (1993) with minor modifications (Onnis et al., 2010). Briefly, buffer containing 1μmol/L hematin, 2mM phenol, 5mmol/L EDTA, substrate (10μmol/L arachidonic acid or 2-AG) and 0.1mol/L tris-HCl, pH 7.4 at room temperature was added to an oxygen electrode chamber with an integral stirring unit (Oxygraph System, Hansatech Instruments, King’s Lynn, U.K., final assay volume 2ml). The oxygen electrode was calibrated with respect to air pressure and ambient temperature. After the addition of 20μl test compound (vehicle, CBD or, as a positive control, flurbiprofen; CBD ± AM630), a baseline was established over a period of 5 min. Reactions were started by addition of ovine COX-1 or human recombinant COX-2 (200U per assay), as appropriate, and the oxygen consumption was measured in 10s blocks. Data are presented as the change in oxygen consumption due to substrate oxygenation (μmol/L) from the point of addition of the enzyme.

Prostaglandin metabolites
The 5ml Krebs’ buffer used to incubate arteries for the 2h incubation and ACh concentration-response curve was collected and frozen at -80°C. In this, using commercially available
enzyme immunoassays (Caymen Chemicals, Cambridge Bioscience, UK), two stable
derivatives of prostaglandin E\textsubscript{2} (13,14-dihydro-15-keto PGA\textsubscript{2} and 13,14-dihydro-15-keto
PGE\textsubscript{2}), and a prostaglandin I\textsubscript{2} (prostacyclin) metabolite (6-keto PGF\textsubscript{1\alpha}) were measured.

Data analysis and statistical procedures
GraphPad Prism (San Diego, CA, USA) was used to plot the data as mean percentage
relaxation, with error bars representing standard error of the mean (SEM), and \( n \) being the
number of arteries from different animals. Data in Figure 1 (where \( n>8 \)) was tested for
normality using the D-Agnostino-Pearson omnibus normality test. The software was used to
fit sigmoidal concentration-response curve to the mean data using a logistic equation.
Percentage maximal vasorelaxant responses (\( \text{R}_{\text{max}} \)) calculated from this mean data were
compared between strains and treatments using unpaired Students’ t-test or one-way
ANOVA followed by Bonferroni’s post hoc tests, with \( p<0.05 \) taken as significant.

Drugs, chemical reagents and other materials
Unless otherwise stated, all chemicals were purchased from Sigma (UK). Apamin, GW9662,
L161982, URB597, O1918, AM251 and AM630 were bought from Tocris (UK). U46619 was
purchased from Enzo Life Sciences (UK). CAY10441, ovine COX-1 and human recombinant
COX-2 were purchased from the Cayman Chemicals (USA). In the myography experiments,
CBD was a gift from GW Pharmaceuticals (UK). For the experiments with purified COX
enzymes, CBD was obtained from Tocris (UK).

Stock solutions of CBD, U46619, GW9662, O1918, indomethacin, nimesulide,
flurbiprofen, CAY10441, AH6809 and HU308 were made to 10mmol/L in ethanol, TRAM-34,
AM251, AM630 and L161982 were made to 10mmol/L in DMSO, and URB597 was made to
1mmol/L in DMSO. Stock solutions of apamin (0.5mmol/L), L-NAME (100mmol/L), PEG-
catalase (100,000U/ml), methoxamine, acetylcholine, sodium nitroprusside and TRAM-34
(all 10mmol/L) were made to in distilled water. DETCA was dissolved directly into Krebs.
Serial dilutions of compounds were made daily.
**Results**

*Cannabidiol improves vasorelaxant responses*

2h incubation with 10µM CBD enhanced the maximum vasorelaxation compared to vehicle (5µl ethanol). In lean ZDF rats, CBD caused about a 15% increase in the maximal response to Ach (R\textsubscript{max} values vehicle 51 ± 2 vs CBD 59 ± 2, p<0.01, unpaired Student t test, Fig 1A). In ZDF rats, CBD caused about a 40% increase in vasorelaxation (R\textsubscript{max} values vehicle 46 ± 1 vs CBD 65 ± 1, p<0.001, unpaired Student t test, Fig 1B). The CBD-induced enhancement of ACh responses was still apparent when the preparations were washed out after the 2h incubation period (p<0.001, Fig 1C). The vasorelaxant response to sodium nitroprusside (SNP) was enhanced in lean but not ZDF rats after 10µmol/L CBD (R\textsubscript{max} values: ZDF lean ethanol-incubated 55 ± 6, n=7; ZDF lean CBD-incubated 72 ± 2, n=7, p<0.05, data not shown).

*Cannabinoid receptor involvement*

CBD-enhanced vasorelaxation was still observed following co-incubation of the arteries with the CB\textsubscript{1} receptor antagonist AM251 (p<0.01, Fig 2A), the FAAH inhibitor URB597 (P<0.01, Fig 2C), the CB\textsubscript{2} antagonist O1918 (p<0.05, Fig 2D) and PPAR\gamma antagonist GW9662 (p <0.001, Fig 2E). However, the CB\textsubscript{1} receptor antagonist AM630 inhibited the effects of CBD (Fig 2B). To assess a potential role for CB\textsubscript{2} receptors in the effects of CBD, the CB\textsubscript{2} receptor agonist HU308 was cumulatively added to pre-contracted arteries. HU308 did not cause vasorelaxation of femoral arteries in control conditions. However, following a 2h incubation with CBD, a concentration-dependent vasorelaxation to HU308 was observed (R\textsubscript{max} 35 ± 4%, Fig 2F), which was not affected by the presence of indomethacin.

*Potential involvement of the endothelial pathways*
The CBD-enhanced ACh response persisted after removal of the endothelium (P<0.0001, Fig 3A), inhibition of nitric oxide (p<0.0001, Fig 3B), or a combination of L-NAME& indomethacin (p<0.0001, Fig 3C). However, in the presence of a combination of inhibitors (apamin, TRAM-34, L-NAME and indomethacin) accepted as blocking EDHF, the effect of CBD was no longer significant (p=0.0829, Fig 3D).

Potential involvement of cyclooxygenases
Indomethacin (Fig 4A), flurbiprofen (Fig 4C) and nimesulide (Fig 4E) abolished the enhancement of ACh-induced vasorelaxation by CBD in ZDF rats. The CBD-induced enhanced ACh response persisted in the presence of the DE/EP prostanoid receptor antagonist AH6809 (P<0.05, Fig 4B) and an IP (prostacyclin) receptor antagonist (CAY10441, P<0.001, Fig 4F). However, the EP4 antagonist L161982 abolished the enhancement of acetylcholine-induced vasorelaxation by CBD (Fig 4D).

Cyclooxygenase activity assays
Arachidonic acid is metabolised by COX-1 and COX-2, and the endocannabinoid 2-AG is metabolised by COX-2 but not COX-1. CBD enhanced the metabolism of arachidonic acid by both COX-1 and COX-2, and that of (2-Arachidonoylglycerol) 2-AG by COX-2 (Fig 5A). In all cases, there was a significant (p<0.0001) interaction time x CBD, indicating that the effect of CBD is dependent upon the incubation time used. For the nine experiments undertaken with 10 µM CBD and COX-1 (Fig 5A and B), this was confirmed by expressing the values at 30 sec and 120 sec (after subtraction of the values at 10 sec due to the short lag phase seen in the assays) as % of the corresponding vehicle controls: for the short incubation time, the mean value for 10 µmol/L CBD was 91% of control (95% confidence interval 63-119%), whereas at the 120 second time point, the corresponding value was 174% (95% confidence interval 140-209%, p<0.005, one-sample t-test).

Because AM630 has a structural similarity to indomethacin, we tested whether this compound might interact with COX-1. On its own, 30µM AM630 inhibited COX-1 activity
AM630 also inhibited the ability of CBD to enhance COX-1 metabolism. The initial inhibitory effect of AM630 on COX-1 activity occurs faster than the ability of CBD to increase COX-1 activity (Fig 5B). Thus, a two-way ANOVA for matching for both CBD and AM630 of the change in oxygen tension between 10 and 30 sec gave F₁,₃(CBD)=0.22, p>0.6; F₁,₃(AM630)=14, P<0.05; F₁,₃(CBD x AM630)=30, p<0.05. The corresponding values for the change in oxygen tension between 10 and 120 sec were F₁,₃(CBD)=10, P<0.05; F₁,₃(AM251)=12, P<0.05; F₁,₃(CBD x AM630)=5.5, P=0.1.

Prostaglandin metabolites

After the Ach concentration response curve, the incubate was tested for prostaglandin metabolites. The amount of PGE metabolites in most of the samples were below the minimum detection limit of the assay (data not shown). However, levels of 6-keto PGF₁α were detectable and ZDF femoral arteries treated with CBD had significantly less 6-keto PGF₁α than the other conditions (Fig 5C).

Potential involvement of hydrogen peroxide and superoxide dismutase

CBD enhanced vasorelaxation to ACh in the presence of PEG-catalase (metabolises hydrogen peroxide) (p<0.01, Fig 6A), or catalase plus L-NAME and indomethacin (p<0.001, Fig 6B). However, the superoxide dismutase inhibitor, DETCA, inhibited the effects of CBD on ACh (Fig 6C).

Mechanisms of CBD-enhanced acetylcholine-induced vasorelaxation in ZDF lean rats

In ZDF lean rats, ACh caused a concentration-dependent vasorelaxation which was enhanced by CBD (Fig 1A). As observed in the ZDF rats, these actions of CBD were blocked by co-incubation with either indomethacin (Fig 7A), nimesulide (Fig 7B), L161982 (Fig 7C), DETCA (Fig 7D), or AM630 (Fig 7E). The effects of CBD were still observed following removal of the endothelium in arteries from the ZDF lean rats (Fig 7F).
DISCUSSION

This study was designed to identify the mechanisms of action underlying the improvements in endothelial function observed in arteries from diabetic rats after prolonged incubation with CBD. CBD enhances COX activity in arteries leading to the production of vasodilator prostanoids acting at the EP4 receptor (see Figure 8 for a summary diagram of the proposed mechanisms of action). This represents another facet in the therapeutic potential of CBD in diabetes, which has already been shown to protect the blood-retinal barrier and endothelial cell activation in hyperglycaemia (El-Remessy et al., 2006; Rajesh et al., 2007), and to decrease insulitis and inflammation, neuropathic pain and myocardial dysfunction in diabetic mice (Weiss et al., 2006, 2008; Rajesh et al., 2010; Toth et al., 2010).

CBD enhances ACh responses in diabetic rats: a role for the endocannabinoid system

Our previous findings that CBD enhances ACh responses (Stanley & Wheal et al., 2013) are confirmed by the current data. This response persisted after washout of arteries, suggesting intrinsic alterations in the arteries. Use of appropriate antagonists ruled out activation of CB1, CB2 or PPARγ by CBD. It should be noted that the size of the enhancement caused by CBD visually appears to be reduced by the CB2 antagonist O1918, although the effect is still significant. This might suggest an involvement of this receptor. However, since the effect of CBD persisted in the absence of an endothelium, this is unlikely.

A proposed mechanism of action for the anti-tumour effect of CBD is via the inhibition of FAAH and consequent increase in local levels of endocannabinoids (Massi et al., 2008). We previously showed that incubation of diabetic arteries with either anandamide or 2-arachidonylglycerol can also improve ACh responses in diabetic rats (Stanley & Wheal et al., 2013), and endocannabinoids themselves are vasorelaxants (Randall et al., 2004), and could therefore explain the effect of CBD. However, when this was tested by co-incubation of arteries with a FAAH inhibitor, the effects of CBD persisted.
Treatment with a CB2 antagonist inhibited the effects of CBD. As CBD does not activate CB2 (Pertwee, 2008), two possible explanations are considered:

a) The antagonism produced by AM630 is an off-target effect. AM630 is widely used as a CB2 receptor antagonist/inverse agonist, and blocks this receptor at mid-nanomolar concentrations (Ross et al., 1999). However, relatively little is known about the interaction of the compound with other targets. Since AM630 shares some of the structural components of indomethacin, we tested whether this compound might also interact with COX-1. Inhibitory effects of AM630 on COX-1 were seen at 30µM, but the effects upon COX-1 were modest at 1 µM, which was the concentration used in the vascular studies. Thus, although we feel it unlikely that the sensitivity to AM630 relates to its action upon COX-1, the present study underlines the importance of using selective (i.e. ≤ 1 µM) concentrations of this compound to produce a complete block of CB2 receptors without producing off-target effects.

b) CBD has allosteric effects upon the CB2 receptor. CBD has allosteric effects at glycine (Foadi et al., 2010) and opioid (Kathmann et al., 2006) receptors. Although many compounds cause allosteric modulation of CB1, less is known about modulation of CB2. To support this theory, we found that CBD was able to induce a concentration-dependent vasorelaxant response to a synthetic CB2 ligand which did not cause vasorelaxation on its own (this is typical of CB2 ligands, see Stanley & O’Sullivan, 2014). Thus CBD must be altering receptor function, rather than increasing local levels of endogenous CB2 agonists. Either way, enhancing CB2 responses, which are typically associated with anti-inflammatory actions, might explain some of the effects of CBD in diabetes.

A role for the endothelium

The CBD-induced effects persisted after removal of the endothelium and nitric oxide production, ruling out these typical mediators of ACh responses. It was of interest that a significant response (20%) to ACh could be observed in arteries of diabetic rats after
removal of the endothelium; more than observed after removal of the endothelium in ZDF lean controls (<5% relaxation). This suggests that smooth muscle mediators of vasorelaxation are upregulated in compensation for endothelial-dysfunction in diabetes, and that CBD can enhance this response. CBD was also able to enhance the vasorelaxant response to the endothelium-independent relaxant SNP, suggesting vascular smooth muscle is a likely target of CBD. Inhibition of endothelium-derived hyperpolarisation almost completely abolished the ACh response in diabetic arteries, and although CBD still appears to enhance the response, this was no longer significantly different to the vehicle control, so a role for endothelium-derived hyperpolarisation cannot be ruled out.

A role for COX

The enhanced ACh response after CBD incubation was abolished in the presence of both COX-1 and COX-2 inhibitors. Cyclooxygenases are homodimeric in structure, but the two monomers behave differently, one acting as the catalytic unit and the other as an allosteric regulator (Yuan et al., 2009). Fatty acids that are not themselves substrates for COX can bind to the allosteric monomers and thereby regulate the catalytic activity of the enzymes. For example, palmitic acid increases the activity of human recombinant COX-2 (Dong et al., 2011). Interestingly, the effects of the fatty acids upon COX-2 are less pronounced at higher arachidonic acid concentrations (Yuan et al., 2009). This may also be the case for CBD, given that the concentration of substrate used in the present study (10 µM) was lower than that of Massi et al. (2008) (75 µM). Whatever the explanation, the present data are consistent with the hypothesis that the vascular effects of CBD seen here are due, at least in part, to a direct activation of COX by the compound. To address this hypothesis, we performed in vitro experiments and CBD was found to increase the rate of oxygenation of arachidonic acid by both COX-1 and -2, and of 2-AG by COX-2. The activation showed some time-dependency, suggesting that CBD associates rather slowly with the COX enzymes. With respect to direct effects upon purified COX-2, the only data available to our knowledge are the studies by Massi et al. (2008) and Ruhaak et al. (2001) using arachidonic
acid as substrate. Massi et al. reported that CBD inhibited ram seminal vesicle COX-1 and human recombinant COX-2-catalysed oxygenation of arachidonic acid at concentrations ≥50 µM, with approximately 50% reductions in activity being seen at a concentration of 1 mM. In contrast, Ruhaak et al. reported that CBD at a concentration of 100 µg/ml (~300 µM) did not affect the production of 14C-prostaglandins following incubation of 14C-arachidonic acid with purified ram seminal vesicle COX-1, and produced an apparent increase in activity in the same assay using COX-2 purified from sheep placental cotyledons.

CBD-induced enhanced ACh response persisted in the presence of IP (prostacyclin) and DP/EP prostanoid receptor antagonists. However, an EP4 receptor antagonist abolished the enhancement of ACh-induced vasorelaxation by CBD. Interestingly, the endocannabinoid anandamide has also been shown cause vasorelaxation of the rat aorta via an arachidonic acid-COX-2 derived metabolite acting at EP4 receptors (Herradon et al., 2007) and we have found that 2-AG relaxes human mesenteric arteries partly through the EP4 receptor (Stanley & O’Sullivan, 2014). EP4 is typically activated by PGE$_2$. Therefore, enzyme immunoassays were performed in order to detect any increases in PGE$_2$ metabolites after CBD. Unfortunately, PGE$_2$ metabolites in all conditions were below the limit of detection, although this does not conclusively rule out an increase in the secretion of PGE$_2$; as the arterial segments were very small compared to the volume of buffer assayed. We did however observe that CBD decreased the level of PGI$_2$ metabolites. As PGI$_2$ can have vasoconstrictor effects through the thromboxane receptor when stimulated by Ach (Liu et al., 2012; 2013), its reduction by CBD might suggest that one of the effects of CBD is to reduce the level of vasoconstrictor prostanoids produced by COX metabolism, thereby enhancing vasorelaxant capacity. The reduction in PGI$_2$ might also reflect preferential metabolism of arachidonic acid into other, yet to be identified, products that activate and cause vasorelaxation via EP4 receptors.

A role for SOD
Some of the vascular effects of cannabinoids are due to increases in SOD activity (O'Sullivan et al., 2005; O'Sullivan et al., 2006). Similarly, in the present study, the effects of CBD were abolished by a SOD inhibitor. One mechanism of SOD is to prevent NO being scavenged by endogenous superoxides; however the effects of CBD were unaffected by inhibition of NO synthase. SOD also catalyses the production of H$_2$O$_2$, which brings about some of the endothelium-dependent vasorelaxant effects of ACh (Matoba et al., 2000). However, the effects of CBD in the present study were not inhibited by catalase, which metabolises H$_2$O$_2$ into water and oxygen thus terminating its biological actions. Increased SOD activity must therefore promote vasorelaxation through other mechanisms. For example, Rajesh et al. (2010) found that CBD restores SOD activity in diabetic mice, associated with reduced lipid peroxides and reactive oxygen species.

**ZDF lean controls**

Although the effect of CBD was more pronounced in the diabetic rats, a small effect of CBD to enhance ACh responses was also observed in the arteries of ZDF lean rats. We therefore carried out a series of experiments identifying that the same mechanisms of action of CBD occur in the ZDF lean rats as were identified in the ZDF rats.

**Conclusion**

CBD enhances vasorelaxant responses (to ACh or a CB$_2$ agonist) in arteries from this rat model of type 2 diabetes. The mechanisms of action involve CB$_2$ receptors and the enhancement of COX and SOD activity. The effect of CBD was endothelium-independent. Products of CBD-enhanced COX activity cause vasorelaxation through activation of vasodilator prostanoid EP4 receptors.
ACKNOWLEDGEMENTS

We thank Dr. Richard Roberts for the use of a myograph. There are no conflicts of interest.

CONTRIBUTION STATEMENT

Participated in research design: Wheal, O’Sullivan, Randall, Fowler

Conducted experiments: Wheal, Cipriano

Performed data analysis: Wheal, O’Sullivan, Cipriano

Wrote or contributed to the writing of the manuscript: Wheal, Cipriano, Fowler, Randall, O’Sullivan.
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Footnotes

AJW and all animal studies were funded by Diabetes UK [Reference: 08/0003822]. CJF would like to thank the Swedish Research Council [grant no. 12158, medicine] and the Research Funds of the Medical Faculty, Umeå University, for financial support.
Legends for Figures

Figure 1. Effects of CBD on vasorelaxant responses to Ach. Arteries from ZDF lean or ZDF rats were incubated for 2h with vehicle or 10μM CBD. Some arteries were washed out following the 2hr incubation period (C). Data are mean ± SEM. Data were compared unpaired Students’ t-test. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2. Cannabinoid receptor involvement. ZDF arteries were incubated for 2h with vehicle or 10μM CBD, and co-incubated with 1μM AM251 (A), 1μM AM630 (B), 1μM URB597 (C), 1μM O1918 (D), 1μM GW9662 (E) or HU308 (F). Data are mean ± SEM. For panels A-E, R_{max} values were compared using an unpaired Students’ t-test. In panel F, R_{max} values were compared using a one-way ANOVA, but those co-incubated with vehicle and indomethacin could not be included in the comparisons due to not being able to fit a curve for those data. *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Nitric oxide and endothelium-derived hyperpolarisation. ZDF Arteries were incubated for 2h with vehicle or 10μM CBD, after endothelium denudation (A), or co-incubated with differing combinations of L-NAME (300μM, B) and indomethacin (3μM, C), and both L-NAME and indomethacin combined with apamin (0.5μM) and TRAM-34 (10μM) (D). Data are mean ± SEM. R_{max} values were compared using an unpaired Students’ t-test. ****p<0.001.

Figure 4. Cyclooxygenase involvement. ZDF arteries were incubated for 2h with vehicle or 10μM CBD, and co-incubated with either 3μM indomethacin (A), 1μM AH6809 (B), 10μM flurbiprofen (C), 1μM L161982 (D), 10μM nimesulide (E) or 100nM CAY10441 (F). Data are mean ± SEM. R_{max} values were compared using an unpaired Students’ t-test. *p<0.05, ***p<0.001.
Figure 5. **CBD and cyclooxygenase activity.** A. Changes in activities of ovine COX-1 (towards 10µM arachidonic acid) and human recombinant COX-2 by CBD. F30 refers to 30µM flurbiprofen, as a positive control. B. Changes in activities of ovine COX-1 by cannabidiol (10µM) and AM630 (30µM). Data shown are mean ± SEM (n=4). C. Prostaglandin I2 metabolite content in the Krebs'-Henseleit buffer used in experiments with arteries taken from lean or ZDF rats that had been incubated for 2h with vehicle or 10µM CBD (mean ± SEM, n=7). Values were compared using a one-way ANOVA plus Bonferroni's *post hoc* test on selected pairs. *p<0.05.

Figure 6. **Hydrogen peroxide and superoxide dismutase.** ZDF arteries were incubated for 2h with vehicle or 10µM CBD, and co-incubated with 300µM PEG-catalase alone (A) or in combination with 3µM indomethacin and 300µM L-NAME (B), or with 300µM DETCA (C). Data are mean ± SEM. R<sub>max</sub> values were compared using an unpaired Students' t-test. **p<0.01, ***p<0.001.

Figure 7. **CBD-enhanced vasorelaxation in ZDF lean rats.** Arteries from ZDF lean rats were incubated for 2h with vehicle or 10µM CBD, and co-incubated with either 3µM indomethacin (A), 10µM nimesulide (B), 1µM L161982 (C), 300µM DETCA (D), 1µM AM630 (E), or endothelium-denuded (F). Data are mean ± SEM. R<sub>max</sub> values were compared using an unpaired Students' t-test. ***p<0.001.

Figure 8. Summary of the proposed mechanism of action of CBD-enhanced vasorelaxation in rats.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

A

Percentage Relaxation (%)

- 5 μl ethanol + 3 μM Indomethacin (n=7)
- 10 μM CBD + 3 μM Indomethacin (n=7)

B

Percentage Relaxation (%)

- 5 μl ethanol + 10 μM Nimesulide (n=7)
- 10 μM CBD + 10 μM Nimesulide (n=7)

C

Percentage Relaxation (%)

- 5 μl ethanol + 1 μM L161982 (n=7)
- 10 μM CBD + 1 μM L161982 (n=7)

D

Percentage Relaxation (%)

- 5 μl ethanol + 300 μM DETCA (n=7)
- 10 μM CBD + 300 μM DETCA (n=7)

E

Percentage Relaxation (%)

- 5 μl ethanol + 1 μM AM630 (n=7)
- 10 μM CBD + 1 μM AM630 (n=6)

F

Percentage Relaxation (%)

- 5 μl ethanol minus endothelium (n=7)
- 10 μM CBD minus endothelium (n=6)
Figure 8.

Cannabidiol

\[ \text{CB}_2 \] \[ \uparrow \text{COX} \] \[ \downarrow \text{PGF}_{1\alpha} \] \[ \uparrow \text{prostanoids} \] \[ \downarrow \text{SOD} \] \[ \uparrow \text{Vasorelaxation} \] \[ \uparrow \text{EP}4 \]