Cannabinoid (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, mounted on a myograph, and incubated with CBD (10 μM) or vehicle for 2 hours. 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 rats (P < 0.01) and especially ZDF rats (P < 0.0001). In ZDF arteries, this enhancement persisted after cannabinoid receptor (CB) type 1, endothelial CB, or peroxisome proliferator-activated receptor-γ antagonist 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, COX-1/2- 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 prostanoit EP4 receptor antagonist. Prostaglandin E2 metabolite levels were below the limits of detection, but 6-keto prostaglandin F1α was decreased after CBD incubation. These data show that CBD exposure enhances the ability of arteries to relax via enhanced production of vasodilator COX-1/2–derived products acting at EP4 receptors.

**Introduction**

Cannabinoid (CBD) is a naturally occurring molecule found in the plant *Cannabis sativa*. Unlike the related molecule Δ⁹-tetrahydrocannabinol (THC), it does not activate cannabinoid receptor (CB) type 1 in the brain, and it 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, nabiximols (Sativex; GW Pharmaceuticals, Salisbury, UK), is currently licensed internationally for the treatment of multiple sclerosis, and CBD alone (Epidiolex; GW Pharmaceuticals) 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 hyperglycemia (El-Remessy et al., 2013). A CBD/THC combination, nabiximols (Sativex; GW Pharmaceuticals), is currently licensed internationally for the treatment of multiple sclerosis, and CBD alone (Epidiolex; GW Pharmaceuticals) was recently approved as an investigational new drug by the Food and Drug Administration in children with medically intractable epilepsy.

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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 ($-\text{cis}-3\text{-}[2\text{-hydroxy}-4\text{-}(1,1\text{-dimethylheptyl}) \text{phenyl}]-\text{trans}-4\text{-}(3\text{-hydroxypropyl})\text{cyclohexanol}$) to CB$_1$ and CB$_2$ 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 (CB$_e$), the transient receptor potential vanilloid 1 receptor, $\alpha_1$-adrenoceptors, $\mu$-opioid receptors, and serotonin receptor type A receptors (Pertwee, 2008). CBD also activates and has physiologic responses mediated by peroxisome proliferator–activated receptor (PPAR)$\gamma$ (O’Sullivan et al., 2009; De Filippis et al., 2011; Esposito et al., 2011). Additional mediators of CBD actions include enzymes capable of metabolizing endogenously produced cannabinoids (termed endocannabinoids), such as lipooxygenases, cyclooxygenase (COX)-2, and fatty acid amid hydrolase (FAAH) (Watanabe et al., 1996; Costa et al., 2004; Massi et al., 2008; Takeda et al., 2011).

We recently reported that in vitro incubation with CBD at 1 or 10 $\mu$M improved vasorelaxation in aortae and femoral arteries of Zucker diabetic fatty (ZDF) rats (Stanley et al., 2013b). The aim of the present study was to determine the mechanisms underlying this effect of CBD in the vasculature of the ZDF rat. We hypothesized that these could involve activation of PPAR$\gamma$ (O’Sullivan et al., 2009) and/or actions mediated by key vascular enzymes, such as COX and superoxide dismutase (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 Laboratories, Wilmington, MA) ($n = 51$) were housed in groups of 2–4 in the University of Nottingham Biomedical Services Unit in a 12-hour light/dark cycle at 22 ± 2°C with access to chow and water ad libitum. Homozygote recessive males ($fa^1fa^1$) develop obesity, hyperlipidemia, fasting hyperglycemia, and type 2 diabetes (ZDF rats; $n = 35$). Wild types (+/+ ) and heterozygous ($fa^1+$/+ ) lean genotypes remain normoglycemic (ZDF lean controls; $n = 16$). At 12–13 weeks of age, the rats were stunned by a blow to the head and killed by cervical dislocation. Postmortem blood glucose concentrations were measured using an Accu-Check Aviva analyzer (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 ZDF lean control rats ($384 \pm 2$ g and $305 \pm 14$ g, respectively (mean ± S.D.; $P < 0.0001$, Student’s $t$ test). Blood glucose levels were also higher in ZDF rats than ZDF lean rats ($22.2 \pm 5.1$ mM and $7.6 \pm 0.7$ mM, respectively; $P < 0.0001$).

Experimental Design. Sections of femoral artery (~2 mm; up to 16 segments of femoral artery per rat) were mounted on a myograph (Danish Myo Technology, Aarhus, Denmark) at a basal tension of 4.9 mN in warmed (37°C), gassed (95% O$_2$/5% CO$_2$), modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 10 mM d-glucose, and 2 mM CaCl$_2$). PowerLab recording systems (A&D Instruments, Abingdon, UK) were used to record changes in tension. Contracture with high-K$^+$ buffer

![Fig. 1. Effects of CBD on vasorelaxant responses to ACh. Arteries from ZDF lean rats (A) or ZDF rats (B) were incubated for 2 hours with vehicle or 10 $\mu$M CBD. Some arteries were washed out following the 2-hour incubation period (C). Data are mean ± S.E.M. Data were compared using unpaired Student’s $t$ test. **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, CBD versus vehicle.](https://example.com/fig1.png)
(62.5 mM NaCl, 59.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, and 2 mM CaCl₂) was used to demonstrate the viability of the mounted vessels. Then arteries were incubated for 2 hours with either 10 μM CBD or vehicle (5 μl of ethanol in 5 ml, 0.1%). Following this, arteries were contracted with an α₁-adrenoceptor agonist, methoxamine (10–100 μM), to reach a stable tone (at least 4.9 mN above basal tone). In some cases, U46619 [(5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid], a thromboxane A₂ mimetic, at up to 100 nM was also added to achieve the required level of tone or to help stabilize tone. Cumulative concentration-response curves to acetylcholine (ACh), sodium nitroprusside (SNP), or the CB₂ receptor agonist HU308 (4-[(1,1-dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol; 1 nM to 30 μM) were constructed.

Potential target sites of action were examined using a range of antagonists (or enzyme inhibitors) acting at PPARγ (GW9662 [2-chloro-5-nitro-N-phenylbenzamide]; 1 μM), CB₁ (AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]; 1 μM), CB₂ (AM630 [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone]; 1 μM), the putative CB₂ (O1918 [1,3-dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1...}

**Fig. 2.** CB receptor involvement. ZDF arteries were incubated for 2 hours with vehicle or 10 μM CBD and coincubated with 1 μM AM251 (A), 1 μM AM630 (B), 1 μM URB597 (C), 1 μM O1918 (D), or 1 μM GW9662 (E). (F) A concentration-response curve to the CB₂ agonist HU308 performed in the presence of CBD and/or indomethacin. Data are mean ± S.E.M. For (A–E), Rₘₐₓ values were compared using unpaired Student’s t test. In (F), Rₘₐₓ values were compared using one-way analysis of variance, but those coincubated 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, CBD versus vehicle.
arginine methyl ester (L-NAME; 300 μM), and FAAH (URB597 (cyclohexylcarboxylic acid 3’-(aminocarbonyl)-[1,1’-biphenyl]-3-yl ester); 1 μM). In some arteries, the intima was rubbed with forceps to remove the endothelium. The role of endothelium-derived hyperpolarization was investigated by inhibition of nitric oxide synthase with N\(^\circ\) nitro-l-arginine methyl ester (L-NAME; 300 μM), inhibition of COX with indomethacin (3 μM), and blockade of small and intermediate calcium-activated potassium channels with apamin (0.5 μM) and TRAM-34 (1-(4-[2-chlorophenyl]biphenylmethyl)-1H-pyrazole; 10 μM), respectively.

The involvement of COX was investigated by coincubation with a nonselective COX inhibitor, indomethacin (3 μM) or flurbiprofen (10 μM), or a COX-2–selective inhibitor, nimesulide (10 μM). The prostaglandin D\(_2\)/prostaglandin E prostanooid receptor antagonist AH6809 (6-isopropoxy-9-xanthone-2-carboxylic acid; 1 μM), the EP\(_4\) receptor antagonist L161982 ([1-(2-[trifluoromethyl]phenyl)-4-[cyclohexylcarbamic acid 3-(4-methylethenyl)-2-cyclohexen-1-yl]benzene]; 1 μM), and the prostaglandin I\(_2\) (IP; prostacyclin) antagonist CAY10441 (4,5-dihydro-2-yl-[4-(1-methylethoxy)phenyl][methyl]phenyl-1H-imadazol-2-amine; 100 nM) were used. To establish any role for hydrogen peroxide and superoxides, arteries were coincubated with polyethylene glycol (PEG)–catalase (300 U/ml) to break down active endogenous hydrogen peroxide or sodium diethyldithiocarbamate trihydrate (DETCA; 300 μM) to inhibit SOD.

**COX Activity Assays.** The method used was that of Meade et al. (1995) with minor modifications (Onnis et al., 2010). Briefly, buffer containing 1 μM hematin, 2 mM phenol, 5 mM EDTA, substrate (10 μM arachidonic acid or 2-arachidonoylglycerol (2-AG)), and 0.1 M Tris-HCl, pH 7.4 at room temperature, was added to an oxygen electrode containing 1 mM I\(_2\), 1.5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl[methyl][-1,1’-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophencarboxamide; 1 μM), and the prostaglandin I\(_2\) (IP; prostacyclin) antagonist CAY10441 (4,5-dihydro-N-[4-[4-(1-methylethoxy)phenyl]methyl]phenyl-1H-imadazol-2-amine; 100 nM) were used. To establish any role for hydrogen peroxide and superoxides, arteries were coincubated with polyethylene glycol (PEG)–catalase (300 U/ml) to break down active endogenous hydrogen peroxide or sodium diethyldithiocarbamate trihydrate (DETCA; 300 μM) to inhibit SOD.

**Prostaglandin Metabolites.** The 5 ml of Krebs buffer used to incubate arteries for the 2-hour incubation and ACh concentration–response curve was collected and frozen at −80°C. In this, using commercially available enzyme immunoassays (Cayman Chemicals, Cambridge Bioscience, Cambridge, UK), two stable derivatives of prostaglandin (PG) E\(_2\) (13,14-dihydro-15-keto PG\(_E_2\)) and 13,14-dihydro-15-keto PG\(_E_2\)) and a PG\(_I_2\) (prostacyclin) metabolite (6-keto PG\(_F_2\)\(_\alpha\) were measured.

**Data Analysis and Statistical Procedures.** GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used to plot the data as mean percentage relaxation, with error bars representing S.E.M. and n being the number of arteries from different animals. Data in Fig. 1 (where n > 8) were tested for normality using the D’Agostino-Pearson omnibus normality test. The software was used to fit the sigmoidal concentration-response curve to the mean data using a logistic equation.

**Fig. 3.** Nitric oxide and endothelium-derived hyperpolarization. ZDF arteries were incubated for 2 hours with vehicle or 10 μM CBD after endothelium denudation (A) or coincubated with L-NAME (300 μM) (B), L-NAME and indomethacin (3 μM) (C), or L-NAME and indomethacin combined with apamin (0.5 μM) and TRAM-34 (10 μM) (D). Data are mean ± S.E.M. R\(_{max}\) values were compared using unpaired Student’s t test or one-way analysis of variance followed by Bonferroni post hoc test, with P < 0.05 taken as significant.

**Drugs, Chemical Reagents, and Other Materials.** Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Dorset, UK). Apamin, GW9662, L161982, URB597, O1918, AM251,
and AM630 were bought from Tocris Bioscience (Bristol, UK). U46619 was purchased from Enzo Life Sciences (Exeter, UK). CAY10441, ovine COX-1, and human recombinant COX-2 were purchased from Cayman Chemical Co. (Ann Arbor, MI). In the myography experiments, CBD was a gift from GW Pharmaceuticals. For the experiments with purified COX enzymes, CBD was obtained from Tocris.

Stock solutions of CBD, U46619, GW9662, O1918, indomethacin, nimesulide, flurbiprofen, CAY10441, AH6809, and RU388 were made to 10 mM in dimethylsulfoxide; TRAM-34, AM251, AM630, and L161982 were made to 10 mM in dimethylsulfoxide; and URB597 was made to 1 mM in dimethylsulfoxide. Stock solutions of apamin (0.5 mM), L-NAME (100 mM), PEG-catalase (100,000 U/ml), methoxamine, ACh, SNP, and TRAM-34 (all 10 mM) were made in distilled water. DETCA was dissolved directly into Krebs. Serial dilutions of compounds were made daily.

**Results**

**CBD Improves Vasorelaxant Responses.** Incubation for 2 hours with 10 μM CBD enhanced the maximum vasorelaxation compared with vehicle (5 μl of ethanol). In ZDF lean rats, CBD caused an ~15% increase in the maximal response to ACh ($R_{max}$ values: vehicle, 51 ± 2%; CBD, 59 ± 2%; $P < 0.01$, unpaired Student’s $t$ test; Fig. 1A). In ZDF rats, CBD caused an ~40% increase in vasorelaxation ($R_{max}$ values: vehicle, 46 ± 1%; CBD, 65 ± 1%; $P < 0.0001$, unpaired Student’s $t$ test; Fig. 1B). The CBD-induced enhancement of ACh responses was still apparent when the preparations were washed out after the 2-hour incubation period ($P < 0.001$; Fig. 1C). The vasorelaxant response to SNP was enhanced in ZDF lean but not ZDF rats after 10 μM CBD ($R_{max}$ values: ZDF lean ethanol-incubated, 55 ± 6%; ZDF lean CBD-incubated, 72 ± 2%, n = 7; $P < 0.05$; data not shown).

**CB Receptor Involvement.** CBD-enhanced vasorelaxation was still observed following coincubation of the arteries with the CB1 receptor antagonist AM251 ($P < 0.01$; Fig. 2A), the FAAH inhibitor URB597 ($P < 0.01$; Fig. 2C), the CB2 antagonist O1918 ($P < 0.05$; Fig. 2D), and the PPARγ antagonist GW9662 ($P < 0.001$; Fig. 2E). However, the CB2...
receptor antagonist AM630 inhibited the effects of CBD (Fig. 2B). To assess a potential role for CB2 receptors in the effects of CBD, the CB2 receptor agonist HU308 was cumulatively added to precontracted arteries. HU308 did not cause vaso-relaxation of femoral arteries in control conditions. However, following a 2-hour incubation with CBD, a concentration-dependent vasorelaxation to HU308 was observed ($R_{max} = 35 \pm 4\%$; 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 and 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 endothelium-derived hyperpolarization factor, the effect of CBD was no longer significant ($P = 0.0829$; Fig. 3D).

Potential Involvement of COXs. 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 prostaglandin D$_2$/prostaglandin E prostanoid receptor antagonist AH6809 ($P < 0.05$; Fig. 4B) and the IP (prostacyclin) receptor antagonist CAY10441 ($P < 0.0001$; Fig. 4F). However, the EP$_4$ antagonist L161982 abolished the enhancement of ACh-induced vasorelaxation by CBD (Fig. 4D).

COX Activity Assays. Arachidonic acid is metabolized by COX-1 and COX-2, and the endocannabinoid 2-AG is metabolized 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-AG by COX-2 (Fig. 5A). In all cases, a two-way analysis of variance matching both time and CBD indicated that there was a significant ($P < 0.0001$) interaction term time $\times$ CBD, indicating that the effect of CBD is dependent on the incubation time used. For the nine experiments undertaken with 10 $\mu$M CBD and COX-1 (Fig. 5, A and B), this was confirmed by expressing the values at 30 and 120 seconds (after subtraction of the values at 10 seconds due to the short lag phase seen in the assays) as percentages of the corresponding vehicle controls: for the short incubation time, the mean value for 10 $\mu$M 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 structural similarity to indomethacin, we tested whether this compound might interact with COX-1. On its own, 30 $\mu$M AM630 inhibited COX-1 activity (Fig. 5B). AM630 also inhibited the ability of CBD to enhance COX-1 metabolism. The initial inhibitory effect of AM630 on COX-1 activity at 10 $\mu$M CBD was 91$\%$ of control (95$\%$ confidence interval, 63–119$\%$), whereas at 120 seconds, the corresponding value was 174$\%$ (95$\%$ confidence interval, 140–209$\%$; $P < 0.005$, one-sample $t$ test).

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activity occurs faster than the ability of CBD to increase COX-1 activity (Fig. 5B). Thus, a two-way analysis of variance for matching for both CBD and AM630 of the change in oxygen tension between 10 and 30 seconds gave $F_{1,3}(CBD) = 0.22 (P > 0.6); F_{1,3}(AM630) = 14 (P < 0.05);$ and $F_{1,3}(CBD \times AM630) = 30 (P < 0.05)$. The corresponding values for the change in oxygen tension between 10 and 120 seconds were $F_{1,3}(CBD) = 10 (P < 0.05); F_{1,3}(AM251) = 12 (P < 0.05);$ and $F_{1,3}(CBD \times AM630) = 5.5 (P = 0.1)$.

**PG Metabolites.** After the ACh concentration-response curves, the incubate was tested for PG metabolites. The amount of PGE metabolites in most of the samples was below the minimum detection limit of the assay (data not shown). However, levels of 6-keto PGF$_{1\alpha}$ were detectable, and ZDF femoral arteries treated with CBD had significantly less 6-keto PGF$_{1\alpha}$ than those in the other conditions (Fig. 5C).

**Potential Involvement of Hydrogen Peroxide and SOD.** CBD enhanced vasorelaxation to ACh in the presence of PEG-catalase (metabolizes hydrogen peroxide) ($P < 0.01$; Fig. 6A) or catalase plus L-NAME and indomethacin ($P < 0.001$; Fig. 6B). However, the SOD inhibitor DETCA inhibited the effects of CBD on ACh (Fig. 6C).

**Mechanisms of CBD-Enhanced ACh-Induced Vasorelaxation in ZDF Lean Rats.** In ZDF lean rats, ACh caused a concentration-dependent vasorelaxation that was enhanced by CBD (Fig. 1A). As observed in the ZDF rats, these actions of CBD were blocked by coincubation with 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 EP$_4$ receptor (see Fig. 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 hyperglycemia (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 et al., 2013b) 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 CB$_1$, CB$_2$, or PPAR$\gamma$ by CBD. It should be noted that the size of the enhancement caused by CBD visually appears to be reduced by the CB$_2$ antagonist O1918, although the effect is still significant. This might suggest an involvement of this receptor. However, because the effect of CBD persisted in the absence of an endothelium, this is unlikely.

A proposed mechanism of action for the antitumor 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-AG can also improve ACh responses in diabetic rats (Stanley et al., 2013b), and endocannabinoids themselves are vasorelaxants (Randall et al., 2004); this could therefore explain the effect of CBD. However, when this was tested by coincubation of arteries with an 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:

1. 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 midnanomolar concentrations (Ross et al., 1999). However, relatively little is known about the interaction of the compound with other targets. Because 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 30 μM, but the effects on 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 on 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.

2. CBD has allosteric effects on 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 that did not cause vasorelaxation on its own (this is typical of CB2 ligands; see Stanley and O’Sullivan, 2014a). 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, which was observed more than 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 that vascular smooth muscle is a likely target of CBD. Inhibition of endothelium-derived hyperpolarization almost completely abolished the ACh response in diabetic arteries, and although CBD still appears to enhance the response, this was no longer significantly different from the vehicle control, so a role for endothelium-derived hyperpolarization 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. COXs 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 on 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 in the study 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 on purified COX-2, the only data available, to our knowledge, are the studies by Massi et al. (2008) and Ruhaak et al. (2011) using arachidonic acid as substrate. Massi et al. (2008) reported that CBD inhibited ram seminal vesicle COX-1– and human recombinant COX-2–catalyzed oxygenation of arachidonic acid at concentrations of ≥50 μM, with ~50% reductions in activity being seen at a concentration of 1 mM. In contrast, Ruhaak et al. (2011) 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.

The 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 and O’Sullivan, 2014b). EP4 is typically activated by PGE2. Therefore, enzyme immunoassays were performed to detect any increases in PGE2 metabolites after CBD. Unfortunately, PGE2 metabolites in all conditions were below the limit of detection, although this does not conclusively rule out an increase in the secretion of PGE2 as the arterial segments were very small compared with the volume of buffer assayed. We did, however, observe that CBD decreased the level of PGl2 metabolites. As PGl2 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 PGl2 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, 2006). Similarly, in the present study, the effects of CBD were abolished by a SOD inhibitor. One mechanism of SOD is to prevent nitric oxide from being scavenged by endogenous superoxides; however, the effects of CBD were unaffected by inhibition of nitric oxide synthase. SOD also catalyses the production of H2O2, 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 metabolizes H2O2 into water and oxygen, thus terminating its biologic 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 that determined 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 CB2 agonist) in arteries from this rat model of type 2 diabetes. The mechanisms of action involve CB2 receptors and the enhancement of COX and SOD activity. The effect of Fig. 8. Summary of the proposed mechanism of action of CBD-enhanced vasorelaxation in rats.
CBD was endothelium-independent. Products of CBD-enhanced COX activity cause vasorelaxation through activation of vaso-dilator prostanoid EP4 receptors.

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Authorship Contributions

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