

The *in vitro* and *in vivo* cardiovascular effects of Δ^9 -tetrahydrocannabinol (THC) in rats made hypertensive by chronic inhibition of nitric oxide synthase.

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Running title: Cardiovascular effects of THC in L-NAME-treated rats.

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Abbreviations/chemical structures : AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CB, cannabinoid; COX, cyclooxygenase; G3, third order branch of the superior mesenteric artery; G0, the superior mesenteric artery; HU210, (6*a*R)-trans-3-(1,1-dimethylheptyl)-6*a*,7,10,10*a*-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo [b,d] pyran-9-methanol; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; THC, Δ⁹-tetrahydrocannabinol; TRPV1, Transient receptor potential vanilloid receptor; U46619, 9,11-dideoxy-9*α*,11*α*-methanoepoxy PGF_{2*α*}; WIN55212,2, (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone.

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ABSTRACT

Evidence suggests that Δ^9 -tetrahydrocannabinol (THC) may have anti-hypertensive effects and that the vasodilator effect of endocannabinoids is enhanced in rats made hypertensive by chronic NO synthase inhibition. The aims of the present study were therefore to investigate whether the *in vitro* and *in vivo* cardiovascular responses to THC are altered by L-NAME treatment. The vasorelaxant effects of THC were enhanced in small mesenteric arteries from L-NAME-treated rats. This enhanced response was not inhibited by CB₁ receptor antagonism (AM251, 1 μ M). Pre-treating vessels with the TRPV1 receptor agonist, capsaicin (10 μ M, 1 h), reduced vasorelaxation to THC to a greater extent in L-NAME-treated than control rats. Inhibition of cyclooxygenase with indomethacin (10 μ M) inhibited THC responses in arteries from L-NAME-treated, but not control rats. In conscious, chronically-instrumented rats, THC (1 mg kg⁻¹ i.v.) caused a pressor effect, with vasoconstriction of the renal and mesenteric vascular beds, and hindquarters vasodilatation. Pre-treatment with AM251 (3 mg kg⁻¹ i.v.) reduced the pressor and vasoconstrictor effects of THC, abolished the hindquarters vasodilatation and revealed a bradycardic response. L-NAME-treated rats showed similar pressor and vasoconstrictor responses to THC, but with bradycardia, and reduced hindquarter vasodilatation. These data show that, *in vitro*, isolated arteries of L-NAME-treated rats show enhanced vasorelaxant responses to THC through an increased sensory nerve component and stimulation of prostanoids. However, *in vivo*, THC causes a CB₁ receptor-mediated pressor effect with hindquarters vascular vasodilatation. There was no evidence of enhanced vasodilator effects of THC in L-NAME-treated animals *in vivo*.

The active ingredient of cannabis, Δ^9 -tetrahydrocannabinol (THC), causes vasorelaxation *in vitro* in a number of different isolated arterial preparations by a variety of mechanisms. The first *in vitro* vascular study showed that THC was capable of relaxing rabbit cerebral arterioles (Ellis et al., 1995) through stimulation of prostaglandins. Subsequent research showed THC-mediated endothelium-independent vasorelaxation of the rabbit superior mesenteric artery (Fleming et al. 1999) and vasorelaxation of the rat hepatic and mesenteric arteries through actions on sensory nerves, although not through the archetypal TRPV1 receptor (Zygmunt et al., 2002). We have recently shown that additional mechanisms underlying vasorelaxation to THC in rat mesenteric vessels include activation of a G-protein coupled receptor, inhibition of calcium channels and activation of potassium channels (O'Sullivan et al., 2005).

THC-based medicines possess interesting therapeutic potential as antiemetics, appetite stimulants, analgesics, and in the treatment of multiple sclerosis, epilepsy and glaucoma (see Ben Mar, 2006). Given the vascular actions of THC (Ellis et al., 1995, Fleming et al., 1999; Zygmunt et al., 2002; O'Sullivan et al., 2005), it is of interest to investigate any therapeutic potential of THC in cardiovascular disease and/or any potential cardiovascular side-effects when used for other conditions. Research in the 1970s found that chronic THC treatment caused larger and longer-lasting hypotension in conscious spontaneously hypertensive rats (SHRs) than in normotensive rats (Kosersky, 1978), and that inhalation of THC caused a greater reduction in blood pressure in patients with glaucoma with high blood pressure than normotensive patients (Crawford & Merritt, 1979). These early studies might indicate a potential anti-hypertensive effect of THC. The first aim of the present study was therefore to investigate the vascular responses to THC in isolated mesenteric vessels, as measured by myography (O'Sullivan et al., 2005), in rats made hypertensive by administration the nitric oxide synthase (NOS) inhibitor N^G nitro-L-arginine methyl ester (L-NAME, 10 mg kg⁻¹ day⁻¹ for 4 weeks) (see Ribeiro et al., 1992; Zatz & Baylis, 1998). It has been previously shown that the vasorelaxant response to the endogenous cannabinoid, anandamide, is enhanced after chronic NOS inhibition (Mendizábal et al., 2001).

Despite the collective evidence of THC-mediated vasorelaxation *in vitro*, the mechanisms underlying any depressor effect of THC *in vivo* remain unclear. In anaesthetised animals, a sustained hypotensive response to various cannabinoid agonists, including anandamide and THC, has been reported (Lake et al., 1997a, b; Wagner et al., 2001; Malinowska et al., 2001). This hypotensive effect can be antagonised by cannabinoid CB₁ receptor antagonists (Lake et al., 1997a; Wagner et al., 2001; Malinowska et al., 2001), and

may involve peripheral prejunctional inhibition of noradrenaline release from postganglionic sympathetic axons (Varga et al., 1996; Niederhoffer & Szabo, 1999), although more recently, cardiac mechanisms have been invoked to explain the lowering of blood pressure (see Pacher et al., 2005 for review). By contrast, the cardiovascular effects of cannabinoids in conscious animals are more complex and do not support the notion that cannabinoids are depressor agents (Stein et al., 1996; Gardiner et al., 2001; Gardiner et al., 2002a; Gardiner et al., 2002b). Indeed, in conscious animals, cannabinoids cause a pressor effect that is CB₁-receptor mediated, and susceptible to ganglion blockade i.e. likely to be due to sympathoexcitation as opposed to sympathoinhibition (Gardiner et al. 2001; 2002b). However, no recent studies have examined the cardiovascular response to THC in conscious animals, and thus it is unknown whether the hypotensive response observed to THC in anaesthetised animals (Lake et al., 1997a,b) is also evident in the conscious state, and if so, whether or not it is associated with vasodilatation. Therefore, the second aim of the present study was to investigate the cardiovascular response to THC (1 mg kg⁻¹, i.v.) *in vivo* in conscious animals. The dose of THC used was chosen to have minimal behavioural effects (Bloom et al., 1997; Varvel et al., 2005) and thus minimise any activity-related changes in cardiovascular status.

Lastly, based on our *in vitro* evidence that vasorelaxant responses to THC are enhanced in vessels obtained from animals treated chronically with L-NAME (see results), the final aim of our study was to measure the cardiovascular responses to THC in conscious L-NAME-treated rats *in vivo*, to test the hypothesis that THC will cause vasodilatation and hypotension in L-NAME-treated hypertensive rats.

Methods

L-NAME treatment protocol

All procedures were approved by the University of Nottingham Ethical Review Committee, and were performed under U.K. Home Office Project and Personal Licence Authority. Male, Sprague-Dawley rats (200-250g) were obtained from Charles River (Kent, UK), and housed in a temperature controlled environment (20-22°C) with a 12 hour light/dark cycle, and allowed food and water *ad libitum*. Animals for *in vitro* studies were housed in pairs, whereas those used in the *in vivo* studies were housed individually after surgery. Animals were assigned to either water drinking (control) or L-NAME drinking (0.1 mg ml⁻¹ solution in water) groups, with water changed every 2-3 days. Animals were treated with L-NAME in their drinking water for 4 weeks (Dunn & Gardiner, 1995). Fluid intake was approximately 75 ml kg⁻¹ day⁻¹ giving an L-NAME dose of ~ 7.5 mg kg⁻¹ day⁻¹. To ensure constancy of L-NAME administration post-surgery for catheterisation and throughout the 4 day experimental protocol, L-NAME was infused intravenously (7.5 mg kg⁻¹ day⁻¹) and all rats were given water to drink.

Vessel preparation

Animals for *in vitro* experimentation were stunned by a blow to the back of the head and killed by cervical dislocation. The aorta, superior mesenteric artery and mesenteric arterial bed were removed rapidly and placed into cold Krebs-Henseleit buffer (composition, mM: NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, CaCl₂ 2, D-glucose 10). From the mesenteric arterial bed, 2 mm segments of third-order branches of the superior mesenteric artery (G3) were dissected free of adherent connective and adipose tissue. G3 vessels were mounted on fine tungsten wires (40 μm diameter) on a Mulvany-Halpern myograph (Myo-Interface Model 410A, Danish Myo Technology, Denmark). The superior mesenteric artery (G0; 3-4 mm in length) and thoracic aorta (4-6 mm long segments) were also cleaned of adherent tissue and mounted on fixed segment support pins using the Multi Myograph system (Model 610M, Danish Myo Technology, Denmark). Tension was measured and was recorded on a MacLab 4e recording system (ADInstruments, UK).

Once mounted, all vessels were kept at 37°C in Krebs-Henseleit buffer and gassed with 5% CO₂ in O₂. The mesenteric vessels were stretched to an optimal passive tension of 5 mN and the thoracic aorta to 10 mN tension (O'Sullivan et al, 2005). All vessels were allowed to equilibrate and the contractile integrity of each was tested by its ability to contract

to 60 mM KCl by at least 5 mN. The vasorelaxant effects of THC were assessed in preparations obtained from L-NAME-treated animals compared with control animals. Arteries (G3, G0 and the aorta) were contracted with methoxamine and the thromboxane mimetic U46619 (O'Sullivan et al., 2005) and once a stable contraction was achieved, the effects of THC (100 nM to 100 μ M) were assessed as cumulative concentration-response curves by addition of THC to the 5 ml organ bath.

The steady state response to THC was taken at each concentration and expressed as the percentage relaxation of the pharmacologically-induced contraction. In G3 vessels, the effects of chronic L-NAME treatment on the vasorelaxant response to other vasorelaxants, acetylcholine (endothelium-dependent) and verapamil (endothelium-independent), were also examined.

The involvement of the cannabinoid CB₁ receptor was assessed using the CB₁ receptor antagonist AM251 (1 μ M, Gately *et al.*, 1996) added to the preparations 10 min before pre-contraction (approximately 30 min before the onset of the THC dose-response curve). The involvement of TRPV1 receptors was assessed by incubating vessels for 1 h with the TRPV1 receptor agonist, capsaicin (10 μ M), to deplete the sensory nerves of vasoactive neurotransmitters, followed by a 20 min washout. The role of prostanoids was investigated by performing experiments in the presence of the cyclooxygenase (COX) inhibitor, indomethacin (10 μ M), present throughout the experiment.

It has been previously shown that L-NAME-treated animals show increased contractile responses in the mesenteric bed (Navarro-Cid et al., 1996; Ruiz-Marcos et al., 2001), and that incubation with THC reduces methoxamine-induced contractile responses (O'Sullivan et al., 2006). We therefore investigated the effects of THC on contractile function in arteries (G3 and G0) obtained from L-NAME-treated rats by constructing concentration-response curves to methoxamine in adjacent segments of arteries 2 h after adding either THC (10 μ M) or vehicle (5 μ l ethanol) to the organ baths.

Surgical Procedures

Two weeks after the onset of L-NAME/water drinking, animals underwent the first surgical procedure for implantation of miniaturised Doppler flow probes. Under general anaesthesia (fentanyl and medetomidine, 300 μ g kg⁻¹ of each, i.p., supplemented as required), probes were sutured around the left renal and superior mesenteric arteries, and around the distal abdominal aorta below the level of the ileocaecal artery allowing measurement of hindquarters flow.

Reversal of anesthesia and provision of analgesia was achieved using atipamezole and nalbuphine (1 mg kg⁻¹ of each s.c.). At least 10 days after probe implantation, and subject to animals passing veterinarian checks, the second stage of surgery, catheterisation, was conducted. Using the same anesthetic regimen, catheters were implanted into the distal abdominal aorta via the caudal artery for measurement of arterial blood pressure and heart rate, and three separate intravenous catheters were placed in the right jugular vein for separate drug administration. At this stage, the wires from the flow probes were soldered into a miniature plug which was fixed to a custom-designed harness worn by the rat. The catheters ran through a protective spring attached to the harness and a counterbalanced lever system, which allowed free movement for the animals in their home cage. Following surgery, the animals were left to recover for 24 hours and were fully conscious and freely moving before experimentation, with free access to food and water. Arterial and venous catheters were connected to fluid-filled, double-channel swivels to allow overnight intra-arterial infusion of heparinised (15 U ml⁻¹, 0.4 ml h⁻¹) saline to maintain catheter patency, and intravenous infusion of saline (control rats; 0.4 ml h⁻¹) or L-NAME (7.5 mg kg⁻¹ day⁻¹). The latter approach was taken to ensure constancy of L-NAME administration in the first 24h post-surgery, when fluid intake would be expected to be lower than normal. Thereafter, L-NAME or saline was infused intravenously for the rest of the 4 day experimental period and all animals were given tap water to drink.

In vivo cardiovascular recordings

Arterial catheters were connected to a fluid-filled pressure transducer and the pressure converted into an electrical signal, and sent via a Gould transducer amplifier (model 13-4615-50) to a customised data capture system (Hemodynamics Data Acquisition System, HDAS), University of Limburg, Maastricht, Netherlands). Signals from the Doppler flow probes were also recorded using HDAS via a Doppler flow meter (Crystal Biotech VF-1 Mainframe fitted with high velocity (HVPD-20) modules). HDAS sampled data every 2 ms, which were averaged every cardiac cycle and stored to disc at 5 second intervals.

Experiments were performed over 4 days. On the first and second day, animals were given either THC (1 mg kg⁻¹ in 0.5 ml vehicle over 20 min) or vehicle (saline with 5% propylene glycol and 2% Tween 80, 0.5 ml over 20 min). On both days, this was preceded by a 30 min infusion of the vehicle for AM251 (saline with 5% propylene glycol and 2% Tween 80). On the third and fourth days, animals were given either THC or vehicle as before,

preceded by a 30 min infusion of AM251 (3 mg kg⁻¹, 1.0 ml). Animals did not receive THC on consecutive days.

Statistical analysis

The concentration of vasorelaxant giving the half-maximal response (EC₅₀) was obtained from the concentration-response curve fitted to a sigmoidal logistic equation with the minimum vasorelaxation set to zero using the GraphPad Prism package (Tep-areenan *et al.*, 2003). Maximal responses and pEC₅₀ (negative logarithm of the EC₅₀) values are expressed as mean ± SEM. The number of animals in each group is represented by *n*. *In vitro* data were compared, as appropriate, by Students's unpaired *t*-test or by analysis of variance (ANOVA) with statistical significance between manipulations and controls determined by Dunnett's *post-hoc* test.

All *in vivo* data were analysed offline using Datview software (University of Limburg, Maastricht, Netherlands). Average values at selected time points were extracted into a customised statistical package (Biomed, University of Nottingham, Nottingham) for subsequent analysis. Non-parametric two-way analysis of variance was used for within-group comparisons (Friedman's test), and Mann Whitney (unpaired) and Wilcoxon's (paired) tests were used for between-group comparisons. A *P* value of <0.05 was taken as significant.

Drugs

For *in vitro* experiments, all drugs were supplied by Sigma Chemical Co. (Poole, UK) except where stated. AM251 was obtained from Tocris (UK). Acetylcholine and methoxamine were dissolved in distilled water. THC, capsaicin and verapamil were dissolved in ethanol at 10 mM with further dilutions made in distilled water. Indomethacin was dissolved first in 100 µl ethanol and then dissolved into the Krebs-Henseleit solution. AM251 was dissolved in dimethyl sulfoxide (DMSO) to 10 mM, with further dilutions in distilled water.

For *in vivo* experimentation fentanyl citrate was purchased from Martindale; medetomidine hydrochloride (Domitor) and atipamezole hydrochloride (Antisedan) were obtained from Pfizer; Du Pont supplied nalbuphine hydrochloride (Nubain). L-NAME was supplied by Sigma and dissolved in tap water. AM251 and THC solutions were made in saline with 5% propylene glycol (Sigma) and 2% Tween 80 (BDH).

Results

In vitro myography

There was no significant difference in the level of tone imposed on arteries between control and L-NAME-treated animals (G3; control 1.26 ± 0.09 g tension, $n=38$ vs L-NAME 1.28 ± 0.10 g tension, $n=34$, G0; control 1.30 ± 0.14 g tension, $n=8$ vs L-NAME 1.45 ± 0.23 g tension, $n=9$, Aorta; control 2.64 ± 0.42 g tension, $n=9$ vs L-NAME 2.56 ± 0.33 g tension, $n=8$). Mesenteric resistance vessels (G3) from animals treated chronically with L-NAME showed enhanced vasorelaxant responses to THC compared to control arteries ($n=8$, $P<0.01$, Figure 1a, Table 1). Vasorelaxation to the endothelium-dependent vasorelaxant, acetylcholine, was not affected by the L-NAME-treatment (Figure 1b). Similarly, vasorelaxation to the calcium channel blocker, verapamil, was not different between arteries from L-NAME-treated and control animals (Figure 1c, Table 1).

To establish whether similar enhancements of the vasorelaxant effects of THC are seen in conduit vessels, the effects of chronic L-NAME-treatment were examined on vasorelaxation to THC in the superior mesenteric artery (G0) and the aorta. In preparations taken from animals treated with L-NAME, the maximal relaxant effect of THC was enhanced in G0 vessels (L-NAME-treated $R_{\max} = 38.2 \pm 4.9$, $n=7$ vs control $R_{\max} = 16.4 \pm 2.8$, $n=9$, $P<0.01$, Figure 2a). In the aorta, in control animals, THC did not cause a vasorelaxant effect (5.1 ± 5.7 % contraction at $100 \mu\text{M}$ THC). By contrast, in L-NAME treated animals, THC caused a small degree of vasorelaxation, although this was not significantly different compared to control (4.0 ± 7.7 % relaxation at $100 \mu\text{M}$ THC, see Figure 2b).

The involvement of the cannabinoid CB_1 receptor in THC-mediated vasorelaxation in G3 vessels was investigated using the cannabinoid CB_1 receptor antagonist AM251 ($1 \mu\text{M}$). AM251 did not affect the vasorelaxant response to THC in arteries from either control animals ($n=7$) or from L-NAME-treated animals ($n=6$, Figure 3a, b). The involvement of sensory nerves in THC-mediated vasorelaxation in G3 vessels was investigated by pre-treating vessels for 1 h with the TRVP1 receptor agonist, capsaicin, to deplete sensory neurotransmitters. In control arteries, capsaicin pre-treatment caused an 8-fold reduction in the potency of THC ($n=6$, $P<0.05$). However, in animals treated chronically with L-NAME, the inhibition of the THC-mediated vasorelaxant response was significantly greater, with a 38-fold decrease in the potency of THC ($n=6$, $P<0.01$, ANOVA). There was no significant difference between the L-NAME and control groups in the vasorelaxant response to THC

after capsaicin pre-treatment ($n=6$). The role of prostanoids in the vasorelaxant responses to THC in G3 vessels was investigated using the non-specific cyclooxygenase inhibitor, indomethacin ($10 \mu\text{M}$, present throughout the experiment). No difference was found in G3 arteries from control animals between responses to THC and the presence and in the absence of indomethacin. By contrast, in arteries from L-NAME-treated animals, the potency of THC was significantly reduced in the presence of indomethacin ($n=6$, $P<0.001$). The vasorelaxant response to THC in the presence of indomethacin in arteries from L-NAME-treated animals was significantly lower than that seen in control animals ($n=6$, $P<0.001$, ANOVA).

In the superior mesenteric artery, the maximum contractile response to methoxamine was significantly increased in L-NAME-treated animals compared with control animals (L-NAME $R_{\text{max}} = 1.82 \pm 0.12$ g tension cf control $R_{\text{max}} = 1.04 \pm 0.15$ g tension, $n=5$, $P<0.01$, ANOVA). This effect was reduced after 2 h incubation with $10 \mu\text{M}$ THC ($R_{\text{max}} = 0.95 \pm 0.06$ g tension, $n=5$, $P<0.01$, ANOVA) to a level not significantly different to the controls (Figure 4a). In small resistance vessels of the mesentery (G3), 2 h incubation with THC significantly reduced the maximal contractile response to methoxamine in L-NAME-treated animals (although this was not significantly raised compared to controls) (L-NAME $R_{\text{max}} = 1.62 \pm 0.10$ g tension cf L-NAME & THC $R_{\text{max}} = 1.08 \pm 0.15$ g tension, $n=6$, $P<0.05$, ANOVA, Figure 4b).

In vivo cardiovascular measurements

Resting cardiovascular variables in the two groups of animals on the four experimental days are presented in Table 2. On each day, L-NAME-treated animals had a significantly higher resting blood pressure, with tendencies for decreased vascular conductance, although this was significant only for mesenteric vascular conductance prior to THC infusion after vehicle infusion, and for hindquarters vascular conductance prior to vehicle infusion after vehicle infusion. The dose of THC used was chosen to have minimal behavioural effects (Bloom et al., 1997; Varvel et al., 2005), and although some acute effects of THC were observed on motor activity and behaviour (increased locomotor activity), the cardiovascular responses to THC reported did not appear to be a result of any changes in behaviour.

Hemodynamic responses to THC

In controls, following vehicle administration (saline with 5% propylene glycol and 2% Tween 80, 0.5 ml over 20 min), there were no changes in heart rate, renal or hindquarters vascular conductances over 4 h, but there was a gradual small fall in mean arterial blood pressure (-5 ± 1 mmHg at the end of 4 h, $P < 0.05$, Friedman's test, data not shown). THC (1 mg kg^{-1}) caused a long-lasting increase in blood pressure compared to baseline values (up to 2 h post-infusion) in control animals (Figure 5). This was accompanied by significant decreases from baseline in renal and mesenteric vascular conductances, and an immediate and long lasting (1h) increase in hindquarter vascular conductance, with a small fall in hindquarters vascular conductance towards the end of the experimental period (Figure 5). Although the fall in heart rate in response to THC in control rats was not significant (Friedman's test, Figure 5), the integrated (0 - 240 min) bradycardia was significantly greater than the effect of vehicle administration (THC 6702 ± 1361 cf vehicle 1277 ± 680 beats, $P < 0.05$, Wilcoxon's test).

In L-NAME-treated animals, following vehicle administration, there were also no changes in heart rate, renal, mesenteric or hindquarters vascular conductances, but there was a significant fall in mean arterial blood pressure during the last hour of the experiment (-10 ± 2 mmHg at the end of 4h, $P < 0.05$, Friedman's test, data not shown). THC (1 mg kg^{-1}) caused a long-lasting increase in blood pressure in L-NAME-treated animals, accompanied by significant decreases from baseline in renal and mesenteric vascular conductances (Friedman's test, Figure 5). THC also caused an increase in hindquarter vascular conductance; however, this was short-lived (Figure 5).

The effects of THC on blood pressure and mesenteric vascular conductance in L-NAME-treated animals were not significantly different to those seen in control animals (Mann Whitney test), but the integrated (0 - 60 min) renal vasoconstriction was significantly smaller in L-NAME-treated rats (control 785 ± 169 cf L-NAME 383 ± 116 % min, $P < 0.05$, Mann Whitney test). The integrated (0 - 60 min) hindquarters vasodilatation was also significantly smaller (control 1586 ± 344 cf L-NAME 626 ± 103 % min, $P < 0.05$, Mann Whitney test).

Effects of CB₁ receptor antagonism on the hemodynamic responses to THC

In control rats, administration of AM 251 (3 mg kg^{-1} i.v., over 30 min) had no significant effects on resting cardiovascular status over the 30 min prior to administration of vehicle or THC. In rats given AM251 followed by vehicle, there was a small fall in mean arterial blood

pressure over time (-5 ± 2 mmHg at the end of 4 h, $P < 0.05$, Friedman's test, data not shown). In rats given AM251, the integrated (0 - 60 min) pressor effect of THC over the first 60 min was diminished compared with THC alone (THC 845 ± 150 cf THC & AM251 389 ± 125 mmHg min, $P < 0.05$, Wilcoxon's test) and the renal and mesenteric vasoconstrictions and the hindquarters vasodilatation were abolished (Figure 6A). In the presence of AM251, THC caused a significant and long-lasting bradycardia (up to 4 h post-infusion, Figure 6A) that was significantly greater than that seen with THC alone (integrated area 0 to 240 min; THC 5219 ± 995 cf THC & AM251 9667 ± 1568 beats, $P < 0.05$, Wilcoxon's test). There was a small decrease in mean arterial blood pressure between 2 and 4 h after administration of THC in animals given AM251 (-9 ± 3 mmHg at the end of 4 h, $P < 0.05$, Friedman's test), but this did not differ from the changes seen with AM251 followed by vehicle administration (see above).

In L-NAME-treated animals, on one of the experimental days where AM251 was given, it caused a small but significant increase in resting blood pressure prior to THC administration (118 ± 3 to 125 ± 2 mmHg after 30 min), accompanied by a decrease in mesenteric vascular conductance (55 ± 5 to 49 ± 4 (kHz mm Hg) 10^3). In the presence of AM251, the effects of THC on blood pressure and mesenteric vascular conductances were markedly inhibited, the hindquarters vasodilatation was abolished, and the hindquarters vasoconstriction was enhanced (Figure 6B). There was no difference between control and L-NAME-treated rats in the response to THC after AM251 administration (Mann Whitney test, Figure 7).

Discussion

This study characterises the cardiovascular responses to THC both *in vitro* and *in vivo* in normotensive and chronic L-NAME-treated rats. Results indicate that, *in vitro*, isolated arteries of L-NAME-treated rats show enhanced vasorelaxation to THC, due to an increased sensory nerve component and stimulation of prostanoid production, but not through the CB₁ receptor. Although THC caused vasorelaxation of mesenteric resistance vessels *in vitro*, *in vivo*, THC caused a CB₁ receptor-mediated pressor effect and mesenteric vasoconstriction, with a CB₁ receptor-mediated increase in hindquarter conductance in normotensive rats. Furthermore, in contrast to the findings *in vitro*, there was no evidence for enhanced vasodilator effects of THC *in vivo* following L-NAME treatment.

The first aim of the present study was to investigate the vascular responses to THC in isolated resistance arteries from rats treated chronically with L-NAME, on the basis that Mendizábal et al. (2001) previously reported that vasorelaxation to anandamide was enhanced in perfused mesenteric beds of these animals. We found that in mesenteric arteries from control rats, THC caused modest vasorelaxation of a similar potency to that previously published (O'Sullivan et al., 2005). However, in rats treated chronically with L-NAME, the vasorelaxant response to THC was significantly enhanced. While it has not been previously examined whether THC responses are altered after chronic NOS inhibition, these data are consistent with previous studies showing that THC causes a greater depressor effect in hypertensive than normotensive patients (Crawford & Merritt, 1979), and that THC causes hypotension in conscious spontaneously hypertensive rats (SHRs) but not in normotensive rats (Kosersky, 1978).

In SHRs, CB₁ receptor antagonists increase blood pressure (Batkai et al., 2004) and CB₁ receptor agonists lower blood pressure more than in normotensive rats (Lake et al., 1997; Batkai et al., 2004). Immunohistochemical evidence also shows CB₁ receptor up-regulation in the aorta of SHRs (Batkai et al., 2004). Although under normal conditions the CB₁ receptor does not participate in the vasorelaxant effects of THC in mesenteric resistance arteries (O'Sullivan et al., 2005), we examined the possibility that the enhanced vasorelaxant response to THC after L-NAME treatment was due to up-regulation of CB₁ receptor function. However, CB₁ receptor antagonism did not affect vasorelaxation to THC in either groups, ruling out this possibility.

Administration of the TRPV1 receptor agonist, capsaicin, leads to a greater depressor effect in SHRs than normotensive rats (Li et al., 2003). Since it is known that THC causes

vasorelaxation through the release of calcitonin gene-related peptide (CGRP) from sensory nerves (Zygmunt et al., 2002), we investigated the role of sensory nerves in vasorelaxation to THC in L-NAME-treated animals. Capsaicin pre-treatment reduced the potency of THC in both groups of animals; however, the inhibition of the THC-mediated vasorelaxant response was greater in the L-NAME-treated animals, indicating that vasorelaxation to THC through sensory nerves may be upregulated in animals treated chronically with L-NAME, potentially through increased release of CGRP or through increased expression of the calcitonin receptor-like receptor (Li et al., 2003).

Vasorelaxation to THC in cerebral arteries is mediated via prostanoids (Eilis et al., 1995), and we investigated whether the enhanced vasorelaxation to THC in arteries from L-NAME-treated rats was due to increased prostanoid release. Although indomethacin did not affect responses in control preparations, in arteries from L-NAME-treated animals, the potency of THC was significantly reduced. COX-2 expression (and vasorelaxant prostanoid production) is enhanced in animals treated chronically with L-NAME in compensation for reduced NO (Henrion et al., 1997). Thus prostanoid release, as a consequence of increased COX-2 expression, may be partly responsible for the increased vasorelaxant effect of THC.

Perfused whole mesenteries from L-NAME-treated animals show increased contractile reactivity (Navarro-Cid et al., 1996; Ruiz-Marcos et al., 2001), possibly due to increased calcium sensitivity (Ruiz-Marcos et al., 2001). We have previously demonstrated that pre-incubation with THC inhibits methoxamine responses, partly through blockade of calcium channels (O'Sullivan et al., 2006), and therefore investigated the effect of THC on contractile responses in L-NAME-treated rats. Importantly, the enhanced contractility of the superior mesenteric artery L-NAME-treated rats was reduced after 2 h incubation with THC to a level not significantly different to control, as has previously been shown with anti-hypertensive treatment (ACE inhibitors or calcium antagonists, Navarro-Cid et al., 1996).

Against the background that THC causes vasorelaxation *in vitro*, the second aim of the present study was to establish whether THC is a depressor agent *in vivo*. We have shown for the first time that in conscious rats, THC causes a rise in systemic blood pressure and vasoconstriction of the renal and mesenteric vascular beds, with vasodilatation in the hindquarters mediated by CB₁. These data contrast with reports on the cardiovascular response to THC in anaesthetised animals, where a sustained, CB₁ receptor-mediated hypotensive response has been reported (Lake et al., 1997a). However, our data are in line with previous reports of pressor effects in response to cannabinoids in conscious rats *in vivo* (Gardiner et al., 2001; Gardiner et al., 2002a; Gardiner et al., 2002b). Our data are also

consistent with those of Jandhyala & Hamed (1978), who showed that the hypotensive effect of THC was observed in anaesthetised but not conscious dogs. Similarly, Lake et al. (1997b) reported a depressor effect of anandamide in anaesthetised, but not conscious, rats.

The hemodynamic response profile to THC is remarkably similar to other CB₁-receptor agonists, which cause renal and mesenteric vasoconstriction with hindquarters dilator actions, also susceptible to AM251, in conscious chronically-instrumented rats (Gardiner et al., 2001; 2002). We have also shown that the pressor response to WIN55212,2 in conscious animals is susceptible to neurohumoral blockade and likely to be due to sympathoexcitation (Gardiner et al. 2001). By contrast, under anaesthesia, cannabinoids inhibit noradrenaline release from postganglionic sympathetic axons, causing hypotension (Varga et al., 1996; Niederhoffer & Szabo, 1999). This debate over cannabinoid-mediated sympathoexcitation versus sympathoinhibition in the conscious versus anaesthetised state may be due to differences in the basal sympathetic tone as it has been previously shown that under anaesthesia, the pressor responses evoked by alpha-adrenoceptor agonist are decreased (Armstrong et al., 1982). Another possibility is that the central effects of cannabinoids might be more susceptible to inhibition by general anaesthesia.

Since THC causes vasorelaxation in isolated arteries, our studies clearly show a discrepancy between the *in vitro* and *in vivo* responses to THC. The results obtained from experiments performed *in vitro* are largely postjunctional and are therefore not influenced by *in vivo* control systems such as the autonomic nervous system. As such, it appears that the CB₁-receptor mediated prejunctional effects of THC (pressor effects) predominate over any direct vascular actions *in vivo*, although, it should be noted that after CB₁-receptor blockade, a hypotensive and/or vasodilator effect of THC was not revealed. However, it should also be noted that *in vitro*, THC causes only a modest vasorelaxant effect at relatively high concentrations, and it is possible that much larger doses of THC would be required *in vivo* to show a vasodilator effect in the mesenteric vascular bed. Clearly such experiments are not feasible in conscious animals due to the profound behavioural effects of higher doses of THC.

THC has been shown to cause vagally-mediated bradycardia in anaesthetised animals (Kawasaki et al., 1980), and repeated THC dosing in man leads to increased vagal activity (Benowitz & Jones, 1981). We observed that after pre-administration of AM251, the bradycardic response to THC was enhanced, suggesting that CB₁ receptors may be coupled to positive chronotropic activity, either centrally or directly. This would be consistent with the notion that the THC-mediated sympathoexcitation described above also involves tachycardia.

Since vasorelaxation to THC was enhanced in arteries from animals treated chronically with L-NAME, the final aim of this study was to establish whether the *in vivo* hemodynamic responses to THC are altered in chronic L-NAME-treated rats. The most notable results from these experiments were that: (i) the pressor and mesenteric vasoconstrictor responses to THC were similar to the control animals (ii) chronic L-NAME-treated rats showed a long-lasting bradycardic response to THC (iii) the hindquarter vasodilator effect of THC in normotensive rats was absent in L-NAME-treated rats.

It has been suggested that CB₁ receptors modulate cardiovascular function in various rat models of hypertension (Batkai et al., 2004). We therefore investigated whether there was any role for CB₁ receptors in the cardiovascular responses to THC in L-NAME-treated rats. AM251 significantly reduced the pressor effects of THC and reduced the mesenteric vasoconstriction, however, there was no evidence of a CB₁ receptor-mediated depressor effect in the L-NAME-treated rats. On one of the days after AM251 was administered in L-NAME-treated rats, arterial pressure was slightly increased (by 7 mmHg), however this is considerably less (~ 30 mmHg increases in blood pressure) than that reported by Batkai et al. (2004) in anesthetised hypertensive rats after CB₁ receptor antagonism.

The bradycardic response to THC was more prominent in L-NAME rats. Studies by Vasquez et al. (1994) indicated that chronic inhibition of NO in rats enhances the bradycardic component of the baroreflex. Later studies showed that this was related to increased responsiveness to acetylcholine in L-NAME-treated rats in compensation for decreased vagal activity (Araujo et al., 1998). In the context of the present study, this suggests that vagal stimulation by THC leads to a greater reduction in heart rate in chronic L-NAME-treated rats as a consequence of enhanced cardiac muscarinic receptor responsiveness.

In the present study, there was a large and long lasting hindquarters vasodilator response to THC in control animals, sensitive to AM251, and consistent with previous data from our group for other CB₁ receptor agonists (Gardiner et al., 2002). In those studies, the β_2 -adrenoceptor antagonist, ICI118551, also inhibited these effects of WIN-55212-2 and HU210, suggesting the vasodilator response to THC also involves β_2 -adrenoceptors. Since we observed that the hindquarter vasodilatation is greatly reduced after L-NAME-treatment, this response to THC may be mediated by NO, consistent with an involvement of NO in β_2 -adrenoceptor-mediated vasodilatation (Gardiner et al., 1991, reviewed by Ritter et al., 2006).

In conclusion, we have shown that although THC causes vasorelaxation *in vitro*, *in vivo*, THC infusion causes a CB₁-mediated pressor effect, which is likely to be due to

sympathoexcitation. Similarly, despite enhanced vasorelaxant responses to THC in arteries from L-NAME-treated rats, the pressor response to THC *in vivo* was similar between control and hypertensive rats. Our results provide no evidence for a hypotensive effect of acute THC administration in either normotensive or L-NAME-treated, hypertensive conscious rats.

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Footnotes

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Figure Legends

Figure 1. The effects of chronic L-NAME treatment on the vasorelaxant response to THC (A), acetylcholine (B) and verapamil (C) in resistance vessels (G3) of the rat mesentery (G3). Data are given as means with error bars representing SEM. * denotes a significant difference between control and L-NAME-treated arteries in the pEC_{50} obtained for the vasorelaxant compounds.

Figure 2. The effects of chronic L-NAME treatment on the vasorelaxant response to THC in the superior mesenteric artery (G0) and the aorta. Data are given as means with error bars representing SEM. * denotes a significant difference between control and L-NAME-treated arteries in the maximal vasorelaxant response (R_{max}) to THC.

Figure 3. The effects of cannabinoid CB_1 receptor antagonism using AM251 (1 μ M) on the vasorelaxant response to THC in resistance vessels of the rat mesentery (G3) in rats treated with vehicle (a) or L-NAME (b). The effects of capsaicin pre-treatment (10 μ M, 1 h) on the vasorelaxant response to THC in control (c) or L-NAME-treated rats (d). The effects of COX inhibition using indomethacin (10 μ M) on the vasorelaxant response to THC in G3 in control (e) or L-NAME-treated rats (f). Data are given as means with error bars representing SEM. * denotes a significant inhibitory effects of compounds on the potency of THC in arteries obtained from control and L-NAME-treated animals.

Figure 4. The effects of chronic L-NAME treatment on the contractile response to methoxamine in the presence and absence of THC (10 μ M) in the superior mesentery artery (A) and the resistance vessels of the mesentery (B). Data are given as means with error bars representing SEM. † denotes a significant difference ($P < 0.05$) between L-NAME-treated and control animals, * denotes a significant difference between L-NAME-treated vessels and L-NAME-treated vessels incubated with THC (10 μ M) for 2 h.

Figure 5. Hemodynamic responses to an infusion of THC (1 mg kg^{-1} infused over 20 min i.v., arrows mark the beginning and end of infusion) in water-drinking (●, closed circles) and L-NAME-drinking (○, open circles) conscious Spague Dawley rats. Data are given as means with vertical bars representing SEM. * denotes a significant change in variables from

baseline (Friedman's Test) within each group in response to THC. Between-group comparisons based on integrated responses are given in the text.

Figure 6. Hemodynamic responses to an infusion of THC (1 mg kg^{-1} infused over 20 min i.v., arrows mark the beginning and end of infusion) in the absence (\bullet , closed circles) and presence (\circ , open circles) of the CB_1 receptor antagonist AM251 (3 mg kg^{-1} i.v. infused over 30 min) in water-drinking (A) and L-NAME-drinking (B) conscious Spague Dawley rats. Data are given as means with vertical bars representing SEM. * denotes a significant change in variables from baseline (Friedman's Test) within each group in response to THC in the presence or absence of AM251. Between-group comparisons based on integrated responses are given in the text.

Figure 7. Hemodynamic responses to an infusion of THC (1 mg kg^{-1} infused over 20 min i.v., arrows mark the beginning and end of infusion) in the presence of AM251 (3 mg kg^{-1} i.v. infused over 30 min) in water-drinking (\bullet , closed circles) and L-NAME-drinking (\circ , open circles) conscious Spague Dawley rats. Data are given as means with vertical bars representing SEM. * denotes a significant change in variables from baseline (Friedman's Test) within each group in response to THC in the presence of AM251. Between-group comparisons based on integrated responses are given in the text.

Table 1. pEC₅₀ values in G3 vessels from control and L-NAME-treated animals. * denotes a significant difference between the control and L-NAME treated animals. † denotes a significant difference from control THC pEC₅₀ values within each group.

| | Water-drinking controls | L-NAME-treated |
|--------------------|-------------------------|-------------------|
| Acetylcholine | 7.64 ± 0.17 | 7.81 ± 0.13 |
| Verapamil | 7.53 ± 0.19 | 7.72 ± 0.25 |
| THC | 5.58 ± 0.12 | 6.13 ± 0.13 ** |
| THC & AM251 | 5.87 ± 0.21 | 6.02 ± 0.16 |
| THC & capsaicin | 4.68 ± 0.30 † | 4.55 ± 0.25 †† |
| THC & indomethacin | 5.82 ± 0.26 | 5.14 ± 0.20 †† ** |

Table 2. Resting cardiovascular variables prior to administration of THC or its vehicle. Data are given as mean \pm SEM. * denotes a significant difference between control and L-NAME-drinking animals in resting variables prior to administration of drugs (Mann Whitney Test).

| Control rats | | | | | |
|----------------------------------|--------------------------------------|-----------------------|---|--------------|--------------|
| Administration protocol | Heart rate (beat min ⁻¹) | Blood pressure (mmHg) | Vascular Conductance (kHz mm Hg)10 ³ | | |
| | | | Renal | Mesenteric | Aortic |
| Vehicle & THC (<i>n</i> =9) | 343 \pm 8 | 110 \pm 2 | 85 \pm 9 | 59 \pm 5 | 39 \pm 5 |
| Vehicle & Vehicle (<i>n</i> =9) | 347 \pm 11 | 110 \pm 3 | 79 \pm 8 | 59 \pm 7 | 40 \pm 2 |
| AM251 & THC (<i>n</i> =8) | 360 \pm 11 | 112 \pm 3 | 77 \pm 7 | 57 \pm 8 | 40 \pm 4 |
| AM251 & Vehicle (<i>n</i> =9) | 352 \pm 10 | 111 \pm 2 | 80 \pm 7 | 56 \pm 6 | 40 \pm 4 |
| L-NAME-treated rats | | | | | |
| Vehicle & THC (<i>n</i> =9) | 331 \pm 6 | 132 \pm 3 * | 74 \pm 6 | 44 \pm 3 * | 30 \pm 3 |
| Vehicle & Vehicle (<i>n</i> =9) | 332 \pm 9 | 129 \pm 3 * | 72 \pm 4 | 48 \pm 4 | 30 \pm 3 * |
| AM251 & THC (<i>n</i> =8) | 341 \pm 10 | 125 \pm 3 * | 78 \pm 4 | 50 \pm 4 | 38 \pm 4 |
| AM251 & Vehicle (<i>n</i> =9) | 326 \pm 10 | 125 \pm 3 * | 78 \pm 5 | 52 \pm 4 | 36 \pm 5 |

Figure 1

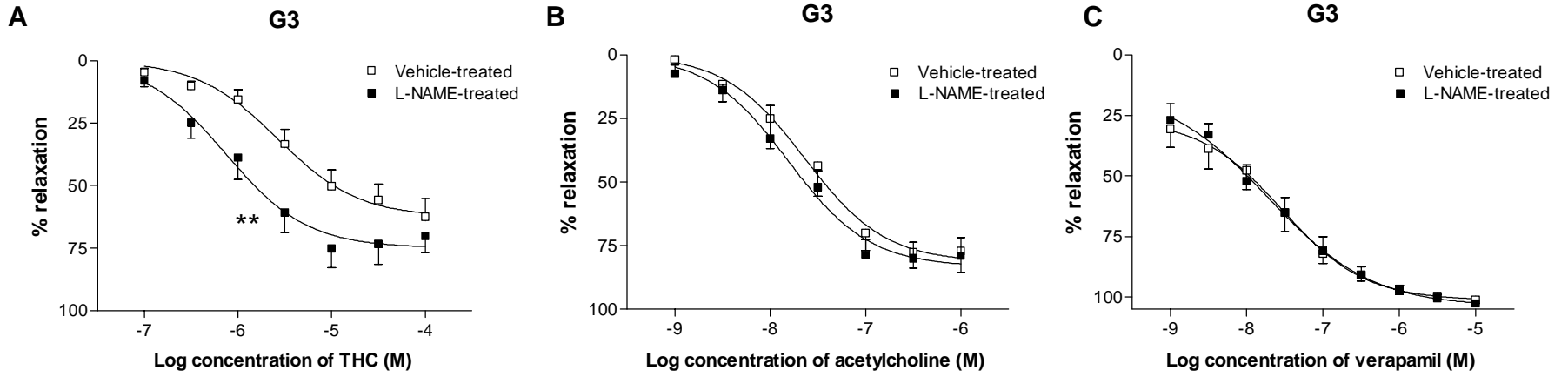


Figure 2

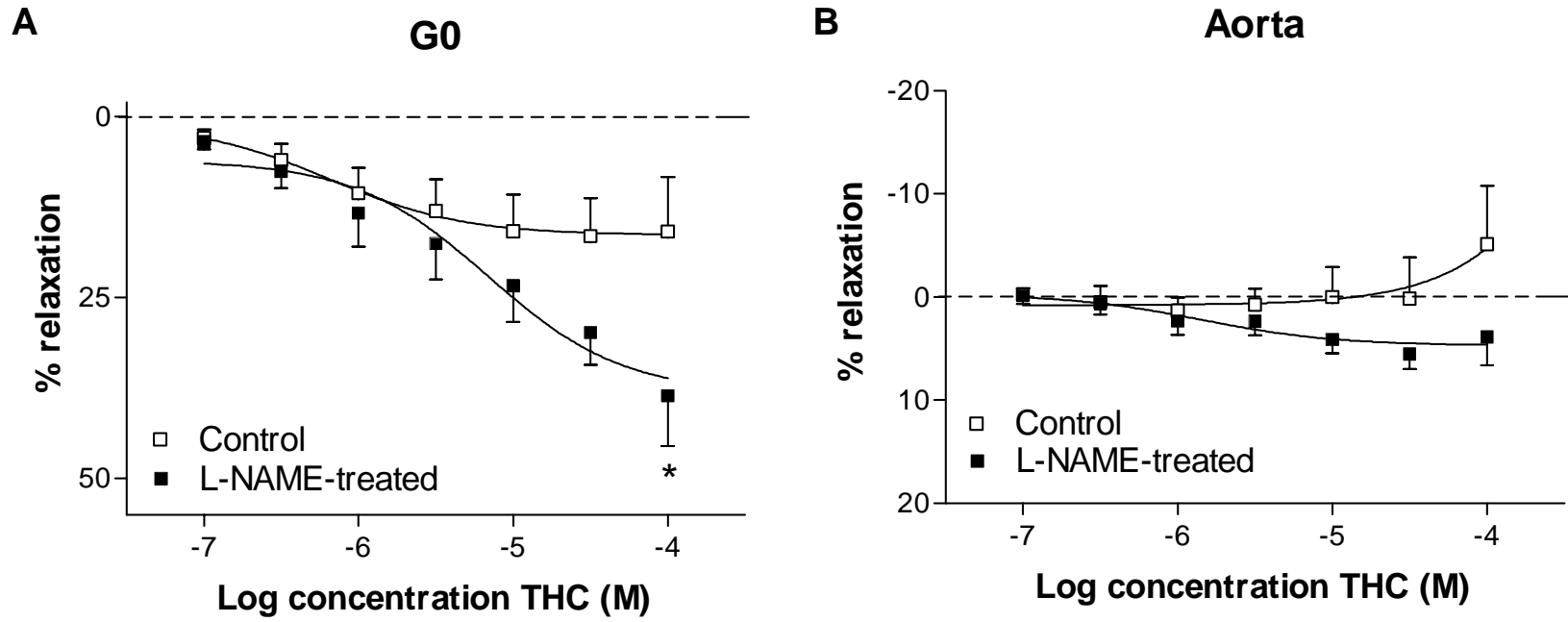


Figure 3

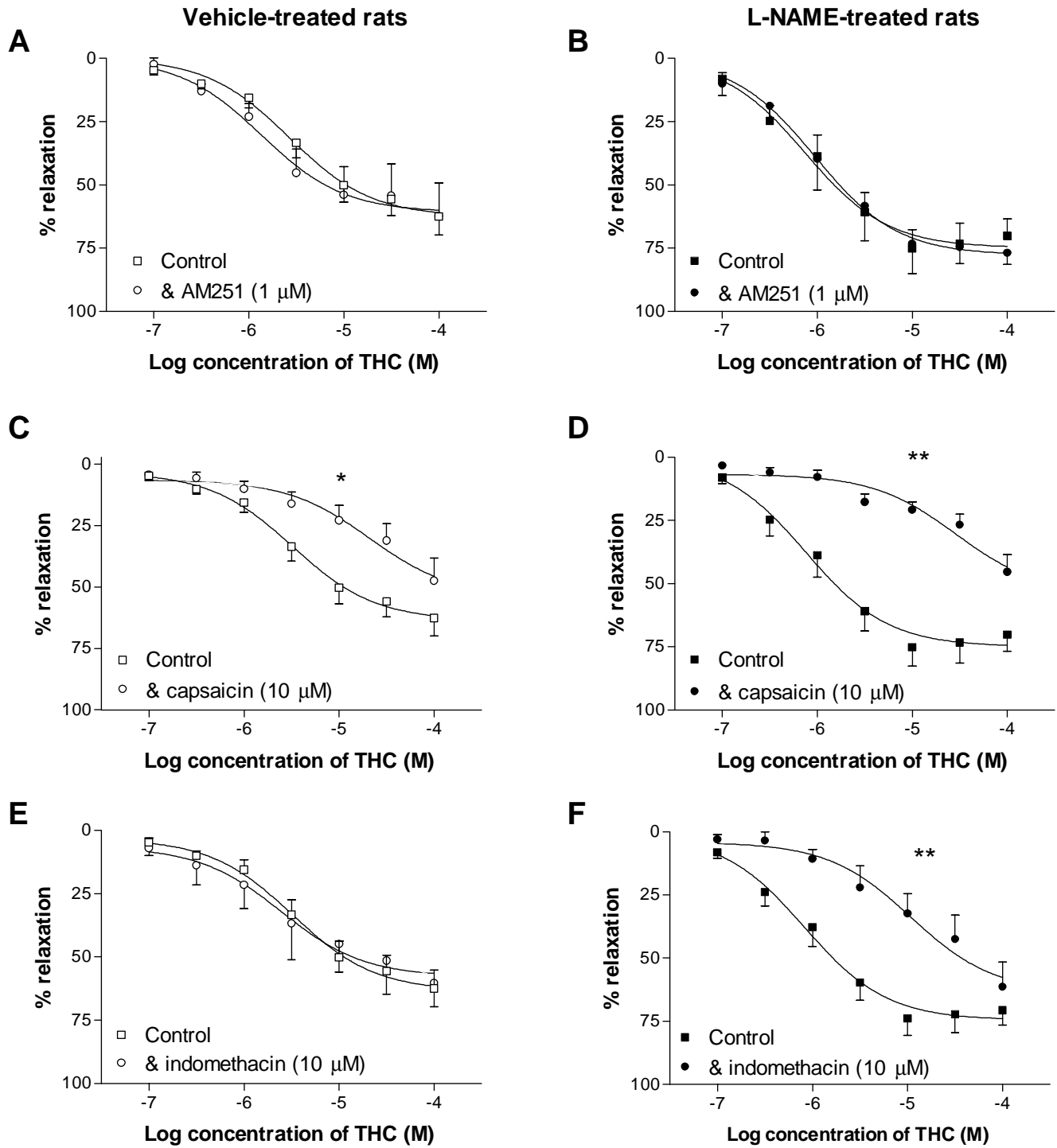


Figure 4

