VASCULAR ACTIONS OF OLEAMIDE IN HEALTH AND DISEASE

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Abstract

Oleamide, an endocannabinoid-like mediator, is a fatty acid that shares structural similarities with anandamide. Oleamide induces cannabimimetic responses and is a potent vasodilator of rat small mesenteric arteries. The cardiovascular actions of oleamide have received relatively little attention in comparison to those of anandamide, the prototypical endocannabinoid. The aim of this study was to examine the vascular effects of oleamide in both health and disease, making a comparison with those of anandamide.

This study demonstrated that oleamide caused vasorelaxation of the rat isolated aorta. The vasorelaxant actions of oleamide were found to be tissue dependent as oleamide did not evoke vascular responses in the porcine mesenteric and coronary arteries. Anandamide did not produce similar responses to oleamide in any of these vessels, displaying marked differences between the two compounds. Oleamide-induced vasorelaxation of the rat aorta was abolished by capsaicin pre-treatment but this was independent of sensory-nerve activity. This demonstrates a potential additional site of action for oleamide and prompted further investigations into the vascular actions of capsaicin. Oleamide also caused relaxation of the rat perfused whole mesenteric arterial bed. This response was diminished by a depolarising concentration of extracellular K⁺, implicating the involvement of K⁺ channels.

Capsaicin evoked relaxation of both rat aortae and porcine coronary arteries. The vasorelaxant effect of capsaicin was insensitive to capsaicin pre-treatment and the presence of capsazepine, a TRPV1 antagonist. It was also found that the presence of capsaicin inhibited the uptake of Ca²⁺ in depolarised porcine coronary arteries and rat aortae on reintroduction of calcium. In porcine coronaries, capsaicin abolished the contractile response to Bay-K 8644, a L-type calcium channel activator. Therefore, it is proposed that capsaicin
inhibits L-type calcium channels to drive vasorelaxation, demonstrating a TRPV1-independent mechanism of action for capsaicin.

Having described the vasorelaxation of Wistar aortae, the effects of hypertension on the vascular actions of oleamide were determined. Oleamide-induced vasorelaxation was significantly enhanced in aortae from spontaneously hypertensive rats (SHR) compared to those from normotensive Wistar Kyoto (WKY) controls. Oleamide caused approximately 40% relaxation of the SHR aorta compared to 15% in the WKY isolated aorta. Similarly, responses to anandamide were also increased in aortae from hypertension causing 30% relaxation compared to 10% in arteries from normotensive controls. Augmented vasorelaxation to oleamide and anandamide was opposed by pre-treatment of vessels with capsaicin, an effect independent of TRPV1 receptors. Inhibition of cyclooxygenase with indomethacin potentiated responses to oleamide in WKY aortae to a level comparable to responses in SHR aortae. Thus, this thesis suggests that changes in the cyclooxygenase pathway are important in regulating responses to oleamide in hypertension and may represent an adaptive change in the early stages of established hypertension in SHR rats.

In summary, this study provides further evidence of the vasorelaxant nature of oleamide, which can be enhanced in arteries from hypertension. Augmented responses in hypertension may relate to alterations in the cyclooxygenase pathway during the early stages of established hypertension in the SHR. This investigation also documents the capsaicin-sensitive nature of oleamide responses in aortic rings, which exists independently of sensory-nerve mediated activity. The observation of a non-TRPV1 capsaicin-sensitive mechanism may ultimately lead to the uncovering of an
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alternative mechanism of action for capsaicin in conduit arteries and a novel site of action for oleamide.
Acknowledgements

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Abbreviations

2-AG 2-arachidonyl glycerol
anandamide  $N$-arachidonylethanolamide
ANOVA analysis of variance
BACAT bile acid-coenzyme A:amino acid N-acyltransferase
$\text{BK}_{\text{Ca}}$ large conductance calcium-dependent potassium-channels
CA1 cornu ammonis area 1
$\text{Ca}^{2+}$ calcium ion
$[\text{Ca}^{2+}]_{i}$ intracellular calcium
cAMP cyclic adenosine monophosphate
CB cannabinoid
CCl$_4$ chemokine (C-C motif) ligand 4
cGMP cyclic guanosine monophosphate
CGRP calcitonin-gene related peptide
CNP C-type natriuretic peptide
CoA Coenzyme A
COX cyclooxygenase
CNS central nervous system
DMSO dimethyl sulphoxide
DRG dorsal root ganglia
DSE depolarisation-induced suppression of excitation
DSI depolarisation-induced suppression of inhibition
EDCF endothelium-derived contracting factor
EDHF endothelium-derived hyperpolarising factor
EDRF endothelium-derived relaxing factor
EET epoxyeicosatrienoic acids
eNOS endothelial nitric oxide synthase
EP$_1$ prostaglandin E receptor 1
EP$_4$ prostaglandin E receptor 4
EtOH ethanol
<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
<tr>
<td>FADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>FALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine gamma thio-phosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>IP</td>
<td>prostacyclin receptor</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium ion</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>Kᵢᵣ</td>
<td>inward rectifier potassium-channel</td>
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<tr>
<td>L-NAME</td>
<td>N⁵-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>MGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NADA</td>
<td>N-arachidonoyl dopamine</td>
</tr>
<tr>
<td>NAE</td>
<td>N-acylethanolamines</td>
</tr>
<tr>
<td>Na⁺-K⁺</td>
<td>sodium-potassium pump</td>
</tr>
<tr>
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</tr>
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<td>N-acyltransferase</td>
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<td>ammonia</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
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<td>N-oleoyl-ethanolamine</td>
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<td>OLDA</td>
<td>N-oleoyl dopamine</td>
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<td>oleamide</td>
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</tr>
<tr>
<td>PAM</td>
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</tr>
<tr>
<td>PE</td>
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<tr>
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<tr>
<td>PGH₂</td>
<td>prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>prostacyclin</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>REM</td>
<td>rapid eye movement</td>
</tr>
<tr>
<td>R_{max}</td>
<td>maximal response</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S.E.M</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rats</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>THC</td>
<td>tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor vanilloid type 1</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
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General Introduction
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Oleamide is an endogenous primary fatty acid amide originally isolated from the cerebrospinal fluid of sleep-deprived cats (Cravatt et al., 1995). Oleamide has been previously demonstrated to induce a range of behavioural effects, including the tetrad of responses used to characterise cannabimimetic activity. Indeed, oleamide is an endogenous agonist at both rat and human cannabinoid CB₁ receptors (Leggett et al., 2004). Endogenous cannabinoids, including the prototypical endocannabinoid anandamide, have received a large amount of interest over the past two decades. Since the isolation of anandamide from porcine brains in 1992, it had been demonstrated to be a potent vasodilator of isolated arteries and has been implicated in the pathophysiology of several cardiovascular disease states. In contrast, the actions of oleamide in the cardiovascular system have received comparatively little interest, thus providing an exciting new area of research. To date, oleamide has been shown to induce potent vasorelaxant responses in rat isolated small mesenteric arteries (Hoi and Hiley, 2006; Sudhahar et al., 2009). The available literature describes a clear overlap in the underlying mechanisms of action responsible for the vascular responses to oleamide and anandamide.

This investigation examined the cardiovascular actions of oleamide in comparison to those of anandamide. Also, the vascular responses to oleamide and anandamide were assessed in a rat model of genetic hypertension, the spontaneously hypertensive rat (SHR).
1.1 Fatty acid amides

Endogenous fatty acid amides represent an important and large group of signalling molecules. Fatty acid amides are alkyl chains comprising 16 or greater carbon atoms with a characteristic single amide moiety and can be broadly categorised into two groups; the primary and secondary fatty acid amides. A major group of the secondary fatty amides are the $N$-acylethanolamines, of which anandamide is the most well known and the prototypical endogenous cannabinoid (CB$_1$) receptor agonist (Devane et al., 1992). The $N$-acylethanolamine group also includes $N$-palmitoyl-, $N$-stearoyl-, and $N$-oleoylthanol-amine (Koga et al., 1997; Mechoulam et al., 1998). Oleamide is one of five primary fatty acid amides isolated from human plasma (Arafat et al., 1989) and was identified in the cerebrospinal fluid of sleep-deprived cats and characterised as cis-9, 10-octadecenoamide (Cravatt et al., 1995). After identification in the cerebrospinal fluid, oleamide has received growing interest as a potentially important signalling molecule. Oleamide has also been demonstrated to be present in rat plasma at 36-57nmol/L (Basile et al., 1999; Driscoll et al., 1999) 35nmol/L in human plasma (Hanus et al., 1999) and 156nmol/L in the cerebrospinal fluid of rats (Hanus et al., 1999). While the natural occurrence of oleamide is apparent, the exact nature of the biosynthetic pathway for oleamide has yet to be fully established.

1.2 Synthesis and degradation of oleamide and anandamide

1.2.1 The biosynthesis of oleamide

Sugiura et al. (1996) described the production of oleamide in brain microsomes using oleic acid and ammonia as the starting substrates. Similarly, the N$_{18}$TG$_2$ neuroblastoma cell line also has the ability to synthesise oleamide from oleic acid (Merkler et al., 2004). The favoured hypothesis for the biosynthesis of oleamide involves the
formation of oleoylglycine that acts as the substrate for peptidylglycine monooxygenase (PAM). The inhibition of PAM in murine N18TG2 neuroblastoma cells blocks the formation of oleamide and increases the production of the upstream substrate oleoylglycine (Merkler et al., 2004). The murine N18TG2 neuroblastoma cell line had previously been demonstrated to express PAM (Ritenour-Rodgers et al., 2000). Thus, Merkler et al. (2004) present strong evidence that the PAM-pathway represents the primary enzymatic route of biosynthesis for oleamide, with oleoylglycine acting as an intermediate substrate. However, this has not been replicated in other cell lines and it also presents another problem as to how oleoylglycine is formed from oleic acid and ammonia. Currently, a number of possible routes for the formation of oleoylglycine have been proposed. Firstly, Burstein et al. (2000) described an oxidative pathway for the formation of N-acyl-glycines involving the actions of alcohol and aldehyde dehydrogenases on oleoylethanolamide. Secondly, it has been proposed that cytochrome c exists as an oleamide synthase (Driscoll et al., 2007). Authors used oleoyl-CoA as a starting substrate because all fatty acids appear to be activated as coenzyme derivatives (Watkins et al., 1997). Driscoll et al. (2007) reported the ability of the rat kidney to synthesise oleamide from oleoyl-CoA and ammonia. In addition, evidence was presented that cytochrome c catalysed the synthesis of oleamide using ammonia as a N-donor in a manner that required hydrogen peroxide as a co-factor (Driscoll et al., 2007). Therefore, cytochrome c may be acting directly on oleoyl-CoA to catalyse the synthesis of oleamide. However, it is also possible that cytochrome c is responsible for the production of oleoylglycine that acts as the downstream substrate for PAM. Indeed, Mueller and Driscoll (2007) detailed the in vitro formation of oleoylglycine by cytochrome c from glycine and oleoyl-CoA. Oleoylglycine and other fatty acid acyl glycines may have important
signalling functions themselves, inducing hypothermia and antinociception in animal models (Chaturvedi et al., 2006).

An alternative enzymatic route proposed for the production of oleoylglycine is via a pathway analogous to that catalysed by bile acid CoA:amino acid N-acyl transferases (BACAT) (Hiley and Hoi, 2007). O’Byrne et al. (2003) described the conjugation of fatty acids to glycine in vitro and also conjugates of bile acids to glycine by BACAT. It is unlikely that BACAT is the authentic enzyme for oleoylglycine synthesis as activity for fatty acids was much lower than for bile acids and it was active at saturated fatty acids, while the proposed starting substrate oleic acid is monounsaturated (O’Byrne et al., 2003).
Figure 1.1 Illustration of the proposed enzymatic pathway for the biosynthesis of oleamide. In summary, oleoylglycine may be activated from its CoA-derivative, oleoyl-CoA, by a cytochrome c complex with glycine as a necessary substrate and hydrogen peroxide as a cofactor. Alternatively, cytochrome c could directly convert oleoyl-CoA into oleamide using ammonia as substrate. It is also possible that the sequential action of alcohol (FADH) and aldehyde (FALDH) dehydrogenase on N-oleoyl-ethanolamide or the action of a BACAT-like enzyme on oleoyl-CoA could provide another route N-oleoylglycine production. Finally, the amidation of N-
oleoylglycine forms oleamide. Figure 1.1 has been adapted from Hiley and Hoi (2007) and Mueller and Driscoll (2009).

1.2.2 The biosynthesis of anandamide

Anandamide is produced on-demand from membrane bound phospholipids as opposed to being released from intracellular stores. A number of biosynthetic pathways have been proposed for the generation of anandamide but the main pathway is considered to be the transacylation-phosphodiesterase pathway (Di Marzo et al., 1994; Sugiura et al., 1996b; 1996c; Cadas et al., 1997). This pathway had originally been demonstrated to be responsible for the synthesis of a range of NAEs (Schmid et al., 1990). The first step of this process involves the formation of N-acylphosphatidylethanolamine (NAPE) from membrane bound phosphatidyl-ethanolamine (PE) (Natarjan et al., 1982; Cadas et al., 1996). N-acyltransferase (NAT) catalyses the addition of a fatty acid acyl to the amino head group of PE from glycerophospholipids, including phosphatidylcholine, in an Ca\(^{2+}\) and cAMP-dependent manner (Liu et al., 2006). The glycerophospholipids act as an acyl donor from the \(S_{n-1}\) position of the glycerol backbone (Jin et al., 2007). The subsequent cleavage of NAPE by phospholipase D forms anandamide and phosphatidic acid. Cadas et al. (1996) demonstrated the Ca\(^{2+}\)-dependent nature of NAT activity, while the rate of the reaction is regulated by cAMP. Indeed, anandamide production is induced in rat neurons by ionomycin, a Ca\(^{2+}\) ionophore (Di Marzo et al., 1994).

1.2.3 Metabolism and uptake of anandamide and oleamide

The removal of anandamide is achieved by its transport into the intracellular space where it is rapidly metabolised. Anandamide can be metabolised by a number of different enzymatic pathways, however, the primary route of degradation is via hydrolysis by fatty
acid amide hydrolase (FAAH) to form arachidonic acid and ethanolamide. The hydrolysis of anandamide was originally reported in neuroblastoma cells and glioma cells (Deutsch and Chin, 1993) but was also demonstrated in rat and porcine brain preparations (Desarnaud et al., 1995; Ueda et al., 1995). Cravatt et al. (1996) identified a membrane-bound hydrolytic enzyme in rat liver cells capable of degrading oleamide into oleic acid. This same enzyme also displayed the anandamide-hydrolysing activity outlined in previous studies and was named FAAH, as it was predicted to act as a general degradative enzyme for fatty acid amides (Cravatt et al., 1995). FAAH is a member of a larger group of enzymes called the amidase signature (AS) family and exists as two different isoforms, FAAH-1 and FAAH-2 (Wei et al., 2006). FAAH-1 has greater affinity for ethanolamides, like anandamide, and is considered to be the primary enzymatic route for anandamide degradation (Wei et al., 2006). FAAH-1 and -2 expression displays both tissue and species specificity. FAAH-1 is highly expressed in the CNS compared to FAAH-2, whereas FAAH-2 is more highly expressed in certain peripheral tissues, including the heart, but is not expressed in rodents for example (Wei et al., 2006).

Studies using FAAH-knockout mice have demonstrated the importance of FAAH activity in the metabolism of anandamide. Mice lacking FAAH exhibited an approximately 15-fold increase in brain levels of anandamide compared to wild-type controls, (Cravatt et al., 2001). FAAH-knockouts also had increased tolerance to pain, an effect that was sensitive to CB₁-receptor antagonism. In addition to hydrolysis, anandamide can also be oxidised directly by the cyclooxygenase (COX)-pathway (Yu et al., 1997; Ross et al., 2002; Weber et al., 2004), lipoxygenase-enzymes (Hampson et al., 1995; Ueda et al., 1995b) and cytochrome P450-dependent systems (Snider et al., 2010; Bornheim et al., 1995).
Before anandamide can be hydrolysed by FAAH situated on the endoplasmic reticulum, it must first be transported across cell membranes and through the cytosol. A number of mechanisms have been proposed to facilitate this cellular uptake. A model of passive diffusion across cell membranes has been put forward, but the majority of the literature suggests the involvement of a facilitated process involving transport proteins. Di Marzo et al. (1994) demonstrated that the uptake of anandamide in cortical neuron cultures is consistent with a carrier-mediated process, namely that uptake is rapid and dependent on temperature and the process is both saturable and selective. Two different forms of facilitated transport of anandamide have been proposed. Firstly, a membrane-bound carrier molecule exists to aid transport across the plasma membrane or, alternatively, that intracellular binding proteins exist to allow movement of anandamide through the cytosol to the site of hydrolysis. In support of this second hypothesis, several proteins have recently been identified that can bind and transport anandamide (Kaczocha et al., 2009; Oddi et al., 2009; Fu et al., 2012).

One of the proposed proteins involved in anandamide transport is a fatty acid binding protein (FABP) (Kaczocha et al., 2009). Previously, a plasma membrane fatty acid has been implicated in the transport of different fatty acids in a range of studies (Berk et al., 1990; 1997; Turcotte et al., 1999; Heather et al., 2006). Anandamide uptake was upregulated by the over expression of FABP5 and FABP7 in N18TG2 neuroblastoma cells and COS-7 cells, which are fibroblast-like kidney cells derived from the monkey (Kaczocha et al., 2009). Furthermore, the authors showed that an additional FABP agonist, in this case oleic acid, attenuated the uptake of anandamide as did the presence of a FABP inhibitor. Oddi et al. (2009) identified albumin and Hsp70 as intracellular proteins capable of binding anandamide by using a biotinylated anandamide analogue, enabling the isolation
and detection of bound proteins by mass spectrometry. In addition, a FAAH-like enzyme lacking hydrolytic activity has the ability to bind and transport anandamide (Fu et al., 2012).

1.3 Evidence for oleamide as an endocannabinoid-like mediator

1.3.1 Cannabinoid receptor pharmacology

The psychoactive nature of marijuana has long been known. Gaoni and Mechoulam (1971) isolated Δ⁹-Tetrahydrocannabinol (THC) as the main psychoactive ingredient of cannabis sativa. However, the mechanisms of action responsible for inducing the powerful psychological effects remained unknown for several decades. Matsuda et al. (1990) cloned the first cannabinoid receptor (CB₁) from the rat brain after the development of selective THC analogues had revealed the existence of cannabinoid binding sites in cerebral cortex of rats (Devane et al., 1988). Subsequently a second cannabinoid receptor (CB₂) expressed in macrophages was identified by sequence homology (Munro et al., 1993). With the discovery of cannabinoid receptors it was hypothesised that an endogenous factor active at these regions would exist. In 1992 anandamide was the first endogenous cannabinoid to be discovered, isolated from the porcine brain (Devane et al., 1992). Anandamide has been demonstrated to bind to CB₁ receptors with Kᵢ values in the nM range (Devane et al., 1992; Vogel et al., 1993; Mechoulam et al., 1995; Adams et al., 1995; Hillard et al., 1995; Showalter et al., 1996; Wise et al., 1996; Petitet et al., 1996). Anandamide exhibits greater binding affinity at CB₁ receptors compared to CB₂ receptors, with Lin et al. (1998) describing a Kᵢ value 3-4 times greater at CB₁ receptors. Subsequently at least four other endocannabinoids have been isolated; 2-AG (Sugiura et al., 1995; Mechoulam et al., 1995), noladin ether (Hanus et al., 2001), virdhamine (Porter et al., 2002)
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and N-arachidonyldopamine (NADA) (Bisogno et al., 2000; Huang et al., 2002).

CB₁ receptors are mainly located in the brain, with high regions of expression in the telencephalon, brainstem, cerebellum, cerebral cortex, spinal cord and dorsal root ganglia (DRG) (Herkenham et al., 1990; 1991; Mailleux and Vanderhaeghe, 1992; Matsuda et al., 1993; Westlake et al., 1994; Glass et al., 1997; Hohnmann and Herkenham, 1999; Farquhar-Smith et al., 2000). CB₂ receptors are present in certain regions of the brain, while expression is high in certain peripheral tissues, particularly immune cells. CB₁-receptor expression has also been described in the cardiovascular system (Gebremedhin et al., 1999; Liu et al., 2000; Batkai et al., 2001; Bonz et al., 2003; Batkai et al., 2004). Liu et al. (2000) demonstrated the existence of functional CB₁ receptors in human endothelial cells from the aorta and hepatic artery. In vascular endothelial cells from human cirrhotic livers the expression of CB₁ receptors was upregulated by approximately 3-fold (Batkai et al., 2001). Expression of CB₁ receptors was also upregulated in the heart and aortic endothelial cells from hypertensive rats (Batkai et al., 2004). Similarly, CB₁-receptor sites were located in human arterial muscle (Bonz et al., 2003).

Both CB₁ receptors and CB₂ receptors are G protein-coupled receptors (GPCRs), which comprise a large group of membrane spanning receptors that initiate intracellular G-proteins to activate or inhibit specific signalling cascades. The cannabinoid receptors couple to the $G_{i/o}$ protein, which acts to inhibit adenylyl cyclase and so regulate intracellular cAMP levels (Vogel et al., 1993; Childers et al., 1994; Deadwyler et al., 1995; Glass et al., 1997; Kern et al., 1999; Glass and Northup, 1999; Childers et al., 2006). Activation of G proteins via CB₁ receptors decreases cAMP levels which can initiate a range of downstream effects. Agonists at CB₁ receptors function to
close Ca\(^{2+}\) channels (Felder et al., 1993; Mackie et al., 1993; Sugiura et al., 1997; Huang et al., 2001; Wang et al., 2003; Brown et al., 2004), activate K\(^{+}\) channels (Mackie et al., 1995; McAllister et al., 1999) and regulate neuronal gene expression (Collin et al., 1995; Terranova et al., 1995; Gerdeman et al., 2002; Ade and Lovinger, 2007). The above effects on intracellular signalling induced by CB\(_{1}\)-receptor activation allow endocannabinoids to act as retrograde signalling molecules.

Previous studies describe CB\(_{1}\)-receptor mediated inhibition of N- and P/Q-type Ca\(^{2+}\) channels (Mackie and Hille, 1992; Caulfield and Brown, 1992; Twitchell et al., 1997; Huang et al., 2001; Liang et al., 2004). Interestingly, Gebremedhin et al. (1999) reported that anandamide and WIN-55,212, a synthetic cannabinoid, induced vasorelaxation of cat cerebral arteries in a manner sensitive to CB\(_{1}\)-receptor inhibition. Also both cannabinoid agonists inhibited L-type Ca\(^{2+}\) currents in isolated cerebral vascular smooth muscle cells, which was identified as the predominant Ca\(^{2+}\) currents in that preparation (Gebremedhin et al., 1999). The cannabinoid CB\(_{1}\) receptor antagonist rimonabant and Pertussis toxin both blocked the inhibitory action of anandamide on L-type calcium currents. Thus, providing a mechanism for anandamide-induced vascular effects via CB\(_{1}\) receptors, although to date CB\(_{1}\)-receptor expression has mainly been located in endothelial cells of the vascular system.

Activation of K\(^{+}\) channels and inhibition of Ca\(^{2+}\) channels results in decreased Ca\(^{2+}\)-influx in neuronal cells and may cause a decrease in neurotransmitter release, possibly underpinning the ability of endocannabinoids to act as retrograde signalling molecules in the regulation of several neuronal processes. For example, endocannabinoid release from depolarised post synaptic cells can act on pre-synaptic cells to activate CB\(_{1}\) receptors to inhibit Ca\(^{2+}\)-influx and suppress GABA release from hippocampal CA1 pyramidal
neurons (Ohno-Shosaku et al., 2002; Yoshida et al., 2002). This action of suppressing GABA-release is termed depolarisation-induced suppression of inhibition (DSI) (Glass and Nunthorpe, 1999). Endocannabinoids also similarly act on pre-synaptic glutamatergic terminals to suppress the release of the excitatory neurotransmitter glutamate, in a process called depolarisation-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001; Maejima et al., 2001) that occurs in Purkinje cells and in dopaminergic cells (Melis et al., 2004).

1.3.2 Oleamide as an endogenous ligand at CB1 receptors

Previous studies have demonstrated that oleamide is a full agonist at CB1 receptors. The binding of both rimonabant and CP55,940 to rat brain membranes was competitively inhibited by oleamide (Leggett et al., 2004). CP55,940 is a synthetic cannabinoid and full agonist of both CB1 and CB2 receptors and is more potent than THC (Griffin et al., 1998; Thomas et al., 1998) CP55,940 binding to human CB1 receptor cell membranes was similarly inhibited by oleamide (Leggett et al., 2004). The authors also described oleamide-evoked \(^{[35]S}\) GTP\(\gamma\)S binding and inhibition of forskolin-stimulated cyclic AMP increases in a manner sensitive to rimonabant. Cheer et al. (1999) similarly demonstrated competitive inhibition of CP55,940 binding by oleamide.

1.3.3. In vitro effects of oleamide

Oleamide evokes a range of behavioural effects, many of which are similar to those induced by anandamide (Mechoulam et al., 1997), including the tetrad of effects used to characterise cannabimimetic activity. The characteristic tetrad of behavioural effects includes hypoactivity, analgesia, hypothermia and catalepsy (Chaperon and Thiebot, 1999).
In rats and mice oleamide evokes hypothermia and hypomobility (Mechoulam et al., 1997; Basile et al., 1999; Yang et al., 1999; Fedorova et al., 2001; Huitron-Resendiz et al., 2001). Indeed, oleamide produced approximately a 2°C drop in the core body temperature of rats (Federova et al., 2001; Murillo-Rodriguez et al., 2001). Oleamide also has analgesic properties, increasing the resistance of rats to pain caused by a beam of light in the tail-flick assay (Fedorova et al., 2001). Other studies report similar analgesia, in addition to catalepsy, in response to oleamide in mice (Mechoulam et al., 1997), although in the rat oleamide was not cataleptic (Federova et al., 2001).

Oleamide levels in cerebrospinal fluid of rats correlates positively with sleep-deprivation and has sleep-inducing properties (Cravatt et al., 1995; Mendelson and Basile, 1999). A 6h period of sleep deprivation caused a 4-fold increase in oleamide concentration in cerebrospinal fluid of rats (Mendelson and Basile, 1999). Herrera-Solis et al. (2010) demonstrated that both oleamide and anandamide administered acutely and sub-chronically, resulted in increased REM sleep, an effect that was blocked by the CB₁ receptor antagonist AM251. A range of other oleamide-induced behavioural effects may also be a consequence of CB₁ receptor activation. Federova et al. (2001) demonstrated that the analgesic nature of oleamide in the tail-flick assay was inhibited by rimonabant. In addition, oleamide can upregulate appetite, a characteristic action of cannabinoids (Dewey, 1986; Martinez-Gonzalez et al., 2004). The importance of the endocannabinoid system in appetite regulation is highlighted by rimonabant-induced weight loss in obese patients (Leite et al., 2009).

While oleamide clearly demonstrates a range of cannabinoid-like pharmacological effects, many are a product of oleamide’s interaction with other neurotransmitter systems. Most notably,
oleamide can interact with the GABAergic and serotonergic systems (Huidobro-Toro and Harris, 1996; Thomas et al., 1997; Yost et al., 1998). Federova et al. (2001) demonstrated that oleamide-induced hypothermia and hypomotility was insensitive to rimonabant. The action of oleamide on locomotion was blocked by the dopamine D_2 receptor antagonist, while a GABA receptor antagonist reversed hypothermia and analgesia (Federova et al., 2001). In CB_1-knockout mice, hypomotility and hypothermia induced by oleamide remained intact (Lichtman et al., 2002).

1.4 Interaction of oleamide and gap junctions

Gap junctions are connecting structures between two cells that facilitate cell-to-cell communication through the diffusion of ions and small molecules (Nicholson, 2003; Martin and Evans, 2004). The coupling of endothelial cells to endothelial cells and to smooth muscle cells has previously been demonstrated and this is thought to be important to vascular function (von der Weid and Beny, 1993; Little et al., 1995; Yamamoto et al., 1998). Gap junctions consist of two hemichannels, called connexons, themselves consisting of six connexin subunits (Goodenough and Paul, 2003). Sandow and Hill (2000) uncovered the existence of myoendothelial gap junctions in rat mesenteric arteries using serial-section electron microscopy techniques. They found an increased incidence of myoendothelial gap junctions in smaller distal arteries in comparison to larger proximal mesenteric arteries (Sandow and Hill, 2000). This corresponds with reports that EDHF-mediated responses were positively correlated with decreasing artery size in the rat mesenteric arterial bed (Shimokawa et al., 1996). It has been demonstrated that gap-junctional communication mediates EDHF responses in mesenteric arteries from the rat, rabbit and guinea pig and also in the rabbit iliac and rat hepatic arteries and in human myometrial arteries (Chaytor et al., 1998; Taylor et al., 1998;
Yamamoto et al., 1999; Edwards et al., 1999; Chaytor et al., 2001; Griffith et al., 2002; Kenny et al., 2002; Chaytor et al., 2005).

Several studies have shown that oleamide can block gap-junctional communication (Guan et al., 1997; Lerner et al., 1997; Boger et al., 1998; Huang et al., 1998; Quist et al., 2000; Bannerman et al., 2000; Boitano and Evans, 2000; Schiller et al., 2001; Krutovskikh et al., 2002; Decrouy et al., 2004; Nagasawa et al., 2006). Indeed, many studies have used oleamide as a pharmacological tool to examine the importance of gap junctions in certain physiological processes. Guan et al. (1997) reported that oleamide blocked communication through gap junctions in rat glial cells, measured by a decrease in electrical conductance and inhibition of dye transfer. Oleamide also caused a decrease in one of the two phosphorylated isoforms of α1-connexin, the primary connexin expressed in glial cells (Guan et al., 1997). However, oleamide did not inhibit Ca$^{2+}$-wave propagation between glial cells although classical gap-junction blockers, such as 18β-glycyrrhetinic acid, were able to (Guan et al., 1997).

Interestingly, oleamide was used to block gap junctions in an investigation examining the role of gap junctions in maintaining the barrier function of porcine blood brain barrier endothelial cells (Nagasawa et al., 2006). Oleamide inhibited the barrier property of tight junction cells in the blood brain barrier. Whether, oleamide actually plays a role in regulating the endothelial blood-brain barrier is not known, however the high concentrations used in this instance are probably not physiologically relevant. One physiological importance of oleamide’s ability to block gap junctions may be in the process of apoptosis (Mueller and Driscoll, 2009). Mueller and Driscoll (2009) proposed a model implicating oleamide in the regulation of cell death. This is based on their observations that cytochrome c can synthesise oleamide (Driscoll et al., 2007) and
that cytochrome c is released from mitochondria during apoptosis (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997). It was postulated that oleamide may act to close gap junctions to regulate cell death and possibly prevent the spread of apoptosis.

Indeed, Krutovskikh et al. (2002) reported that cell coupling through gap junctions propagated the spread of apoptosis in a cancerous rat bladder cell line. In the presence of the gap-junction blocker β-glycyrrhetinic acid and in cells expressing a mutant connexin43 the spread of cell death was decreased. Oleamide inhibited the spread of Lucifer yellow dye in these cells and was used to assess the importance of Ca$^{2+}$-wave propagation, making use of its selectivity on gap junction permeability described in previous studies (Guan et al., 1997; Krutovskikh et al., 2002). However, oleamide itself had no effect on the spread of cell death.

1.5 Cardiovascular effects of the endocannabinoid anandamide

1.5.1 In vitro effects of anandamide

Anandamide has been shown to be a potent vasodilator of blood vessels isolated from different species and vessel beds (reviewed Randall et al., 2002). The mechanisms responsible for the vasorelaxant properties of anandamide appear to be both tissue and species specific. The earliest study showed that anandamide dilated cerebral arteries from rabbits via the cyclooxygenase pathway as the response was sensitive to the presence of indomethacin (Ellis et al., 1995). Anandamide also induces vasorelaxation of rat mesenteric arteries partly in a manner sensitive to non-selective inhibition of the cyclooxygenase pathway with diclofenac (Fleming et al., 1999). Anandamide has since been shown to cause vasodilatation in a range of isolated vessels in a COX-independent manner; basilar artery from the guinea pig (Zygmunt et al., 1999),
bovine coronary artery (Pratt et al., 1998), rabbit aorta (Mukhopadhyay et al., 2002), rat renal artery (Deutsch et al., 1997), rat mesenteric artery (White and Hiley, 1997) perfused rat heart (Fulton and Quilley, 1998; Randall and Kendall, 1997a), rabbit mesenteric arteries (Chaytor et al., 1999) and the perfused rat mesenteric arterial bed (Randall et al., 1996; Randall and Kendall, 1997b; Harris et al., 2002). A number of mechanisms have been implicated in anandamide-induced responses that include sensory nerve activity (Zygmunt et al., 1999), the release of nitric oxide (Deutsch et al., 1997; Bilfinger et al., 1998), gap-junctional communication (Chaytor et al., 1999; Harris et al., 2002), ATP-sensitive K⁺ channels (Chataigneau et al., 1998), Ca²⁺-activated K⁺ channels (Plane et al., 1997; Randall and Kendall, 1998) and EDHF-mediated mechanisms (Chaytor et al., 1999; Harris et al., 2002; O’Sullivan et al., 2004). The tissue specific nature of anandamide-induced is probably best highlighted by O’Sullivan et al. (2004). The authors demonstrated mechanistic differences underlying responses between small resistance and large conduit rat mesenteric arteries. In smaller arteries, anandamide-induced relaxation was attributed to CB₁ receptors and also to EDHF-mediated activity, which was possibly facilitated by gap-junctions and coupled to a novel endothelial cannabinoid receptor. Vasorelaxation of larger conduit arteries was caused by CB₁ receptors and sensory-nerve mediated activity in an endothelium-independent manner (O’Sullivan et al., 2004).

1.5.2 Anandamide and sensory-nerve activation

Zygmunt et al. (1999) suggested the involvement of sensory-nerve mediated activity in anandamide-induced vasorelaxation. Anandamide elicited vasorelaxation of rat isolated hepatic and small mesenteric arteries and guinea-pig basilar artery, all of which were sensitive to capsaicin pre-treatment designed to deplete vasoactive
neuropeptides from perivascular sensory nerves (Zygmunt et al., 1999). The vasorelaxant responses to anandamide were also sensitive to CGRP receptor antagonism, characterising CGRP as the neuropeptide released by activation of capsaicin-sensitive vanilloid receptors by anandamide (Zygmunt et al., 1999). The capsaicin-sensitive vanilloid receptor has been identified as the transient receptor vanilloid type 1 (TRPV1) that functions as a non-selective cation channel regulating permeability to Ca\(^{2+}\). Thus, the activation of TRPV1 causes the increase in intracellular Ca\(^{2+}\) resulting in the release of neuropeptides such as CGRP, substance P and tachykinins, which can regulate vascular smooth muscle. In addition to capsaicin and anandamide, TRPV1 is a target for a range of endogenous and external agonists including lipoxygenase metabolites (Hwang et al., 2000; Sexton et al., 2007), noxious heat (Brauchi et al., 2006) and other fatty acid amides. N-oleoyl-ethanolamine (OEA), N-arachidonoyl dopamine (NADA) and N-oleoyl dopamine (OLDA) have all been shown to interact with vanilloid receptors (Huang et al., 2002; Chu et al., 2003; Movahed et al., 2005; Sharkey et al., 2007). Interestingly, the vasorelaxation of rat small mesenteric arteries induced by oleamide also involved the activation of TRPV1 receptors on sensory nerves. This response was attenuated by the TRPV1 antagonist capsazepine and the cation channel blocker ruthenium red (Sudhahar et al., 2009).

1.5.3 Nitric Oxide release

The characterisation of endothelium-dependent vasorelaxation led to the discovery of an endothelium-dependent relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). This study illustrated the necessity of an intact endothelium in the vasorelaxation of isolated arteries in response to acetylcholine, bradykinin and histamine (Furchgott and Zawadzki, 1980). EDRF was subsequently identified as nitric oxide (NO) (Ignarro et al., 1987a; 1987b; Palmer et al.,
1987). NO had previously been demonstrated to be a potent vasorelaxant in the bovine coronary artery, in which NO was shown to activate soluble guanylate cyclase (Greutter et al., 1979; Ignarro and Kadowitz, 1985).

It was proposed that a cytosolic endothelial enzyme was responsible for the synthesis of an EDRF from L-arginine (Mayer et al., 1989). Indeed, three isoforms of nitric oxide synthase (NOS) have been isolated; neuronal-, endothelial-, and inducible-NOS. Neuronal nitric oxide was identified from brain tissue (Bredt and Snyder, 1990) and a version of this enzyme was found to be constitutively expressed in endothelial cells of bovine aortae (Pollock et al., 1991). Endothelial NOS is stimulated in response to calmodulin after an increase in intracellular Ca\(^{2+}\) (Brendt and Snyder, 1990; Fostermann et al., 1991; Michel et al., 1997). Endothelium-dependent relaxants and shear stress both cause increases in endothelial cell Ca\(^{2+}\) and release of NO (Koller et al., 1994; Fleming et al., 1998). Following synthesis in the endothelium, NO is free to diffuse to the vascular smooth muscle where it can activate guanylyl cyclase by binding to its haem group (Gerzer et al., 1982; Ignarro et al., 1982; Wolin et al., 1982). Activation of guanylyl cyclase upregulates the second messenger 3’, 5’-cyclic guanosine monophosphate (cGMP), which can stimulate cGMP protein kinases (PKG) (Lucas et al., 2000). PKG can activate myosin light chain phosphatase, attenuating the phosphorylation of myosin light chain to initiate the relaxation of vascular smooth muscle (Surks et al., 1999; Torrecillas et al., 2000).

NO is continuously released from the endothelium (Vallance et al., 1989; Stamler et al., 1994) and constitutes an important signalling molecule regulating vascular tone. NO has been demonstrated to be released in response to shear stress causing flow-dependent vasodilatation (Buga et al., 1991). The importance of NO signalling is further highlighted by studies showing that murine knockouts for
endothelial NOS or cGMP activated protein kinases develop hypertension (Huang et al., 1995; Pfeifer et al., 1998). NO has a range of cardioprotective properties and down-regulation of NO production has been implicated in the pathophysiology of a number of cardiovascular disorders, including hypertension, diabetes and hypercholesterolaemia (reviewed in Naseem, 2005).

Due to its importance as a vascular signalling molecule, NO was proposed as a candidate mediating vasorelaxation to endocannabinoids. In support of this, anandamide has been demonstrated to elicit the release of NO (Deutsch et al., 1997; Bilfinger et al., 1998; Poblete et al., 2005). Deutsch et al. (1997) described anandamide-induced vasodilatation of perfused juxtamedullary afferent arterioles from the rat kidney, which was sensitive to inhibition of NO synthase with L-NAME and to rimonabant. In perfused arterial segments from the kidney and in cultured renal microvascular endothelial cells, anandamide stimulated the release of NO (Deutsch et al., 1997). Similarly, anandamide evoked endothelial NO release in the saphenous vein, right atria and thoracic artery in a CB₁-receptor mediated mechanism (Bilfinger et al., 1998). The endocannabinoid 2-AG elicited NO release in human saphenous vein via a process coupled to CB₁-receptor activation (Stefano et al., 2000). In a range of immune cells 2-AG and anandamide caused the release of NO, again via CB₁ receptors (Stefano et al., 1996; 2000). In addition to being able to evoke NO by stimulating endothelial CB₁ receptors it has been shown that the interaction of anandamide with TRPV1 may also release NO. Perfusion of the rat mesenteric arterial bed with anandamide stimulated an acute release of NO. This response to anandamide was sensitive to antagonism of TRPV1 with capsazepine and was also reduced by the presence of rimonabant or AM251 (Poblete et al., 2005). This TRPV1-dependent component of NO
release corresponded with the endothelial expression of TRPV1 mRNA as measured using RT-PCR (Poblete et al., 2005).

1.5.4 Vascular responses to anandamide and vasoactive prostanoids

In addition to NO, the endothelium also releases vasorelaxant prostanoids (Moncada et al., 1976; Moncada et al., 1979; Dusting et al., 1977; Hong, 1980). The endothelium releases a number of different vasoactive prostanoids; prostaglandin H$_2$, I$_2$ (prostacyclin), D$_2$, F$_{20}$, E$_2$ and thromboxane A$_2$. These prostanoids are arachidonic acid derivatives produced via the cyclooxygenase (COX) pathway. The COX-pathway consists of COX-1 and COX-2 enzymes that are expressed in the endothelium and vascular smooth muscle (De Witt et al., 1983; Doroudi et al., 2000; Onodera et al., 2000; Kawka et al., 2007). COX-enzymes convert arachidonic acid, liberated from membrane phospholipids by phospholipases, into PGH$_2$ and PGG$_2$. PGH$_2$ produced by COX is subjected to further transformation by a range of specific prostaglandin synthases. PGI$_2$ synthase is highly expressed in endothelial cells (Siegle et al., 1994; Tone et al., 1997; Kawka et al., 2007) and as such prostacyclin is one of the most abundant of the endothelium-derived prostanoids. Prostacyclin has been demonstrated to be a potent vasodilator of blood vessels (Bunting et al., 1976; Lamontagne et al., 1992; Jackson et al., 1993), causing the relaxation of vascular smooth muscle by interacting with IP receptors (Coleman et al., 1994). Other endothelium-derived prostanoids, including PGH$_2$ and thromboxane A$_2$, have been shown to cause vasoconstriction (Shirahase et al., 1988; Ge et al., 1995; Rapoport and Williams, 1996; Gluais et al., 2006).

Initial investigations implicated the COX-pathway in the vasodilator actions of anandamide. Vasodilatation of rabbit cerebral arterioles to both anandamide and THC was sensitive to inhibition of the COX-pathway with indomethacin (Ellis et al., 1995). This suggests that
metabolism of anandamide or its metabolites by COX results in vasoactive prostanoid production. In rabbit mesenteric arteries and sheep coronary arteries anandamide-induced vasorelaxation was also sensitive to COX inhibition (Fleming et al., 1999; Grainger and Boachie-Ansah, 2001). In addition, Herradon et al. (2007) described an indomethacin-sensitive vasorelaxant response in rat aortae to anandamide. Vasorelaxation of aortae was also attenuated by the FAAH inhibitor URB597, a selective COX-2 inhibitor DFU, and an EP₄ receptor antagonist. Taken together these results predict the degradation of anandamide by FAAH to arachidonic acid, which itself is metabolised by COX-2 to form a vasoactive prostanoid, possibly prostaglandin E₂, active at EP₄ receptors to cause aortic vasorelaxation (Herradon et al., 2007).

In contrast to Herradon et al. (2007), it has been demonstrated that aortic responses to THC, anandamide and NADA were augmented by the presence of indomethacin (O'Sullivan et al., 2005). A picture has emerged in subsequent studies in which the COX-pathway may have a role in limiting the vascular actions of endocannabinoids in some tissue preparations (Ho and Randall, 2007; Wheal et al., 2010). Ho and Randall (2007) demonstrated that in mesenteric vasculature from the rat the relaxant effects of anandamide and 2-AG are regulated by the activity of COX and endocannabinoid hydrolases. Vasorelaxant responses to anandamide were potentiated by inhibition of COX-2 and FAAH. While, 2-AG-induced relaxation was sensitive to inhibition of MGL and COX-1. Similarly, indomethacin also potentiated relaxation to the endocannabinoid-like fatty acid amide N-oleylethanolamine in rat mesenteric arterial beds (Wheal et al., 2010).

1.5.5 Anandamide and the release of an EDHF

As outlined above the endothelium is important in regulating local vascular tone as it is the site for the synthesis and release of the
vascular NO and prostacyclin (Dusting et al., 1977; Palmer et al., 1987). The existence of another as yet unidentified vasoactive mediator released by the endothelium has been demonstrated, which functions independently of NO and prostacyclin release (Taylor and Weston, 1988). This endothelium-dependent relaxant factor is characterised by the hyperpolarisation of vascular smooth muscle, is abolished by depolarising concentrations of K⁺ and is sensitive to a specific combination of K⁺ channel blockers and hence is known as endothelial dependent hyperpolarising factor (EDHF). EDHF-mediated responses are blocked by the combination of apamin and charybdotoxin (Zygmun and Hogestatt, 1996; Corriu et al., 1996). Apamin selectively blocks small conductance Ca²⁺-activated K⁺ channels, while charybdotoxin is used to block both intermediate and large conductance Ca²⁺-activated K⁺ channels. This taken together with the fact that iberiotoxin, an inhibitor of large conductance Ca²⁺-activated channels, does not replicate the effects of charybdotoxin in blocking EDHF-mediated responses, demonstrates that the EDHF response involves small and large Ca²⁺-activated K⁺ channels (Zygmun and Hogestatt, 1996). EDHF-mediated responses involve activation of endothelial Ca²⁺-activated K⁺ channels, caused by an increase in intracellular calcium in response to ligand binding. The resulting efflux of K⁺ into the intercellular space drives the hyperpolarisation of endothelial cells. This hyperpolarisation is effectively passed on from the endothelium to the vascular smooth muscle by an EDHF. A number of different mechanisms and mediators have been proposed to be an EDHF, including gap-junction communication (Chaytor et al., 1998; 2001; Berman et al., 2002; De Vriese et al., 2002; Griffith et al., 2002; Lang et al., 2007) K⁺ ions (Edwards et al., 1998; Coleman et al., 2001), epoxyeicosatrienoic acids (Adeagbo and Henzel, 1998; Miura and Guttermann, 1998; Fleming et al., 2001; Halcox et al., 2001), hydrogen peroxide (Beny and von der Weid, 1991; Matoba et al.,
2002; 2003; Shimokawa et al., 2005) and C-type natriuretic peptide (Chauhan et al., 2003; Villar et al., 2007).

Various investigations have implicated EDHF-mediated mechanisms in the vascular actions of anandamide. Chaytor et al. (1999) described an endothelial component of the vasorelaxation of rabbit superior mesenteric arteries to anandamide that was independent of NO and prostanoid production. The anandamide-induced endothelium-dependent relaxation was sensitive to the gap junction inhibitor 18α-glycyrrhetinic acid, the selective peptide gap-junction inhibitor gap 27 and a concentration of rimonabant (10µM) sufficient to block gap-junction communication (Chaytor et al., 1999). This implicates gap-junctions and therefore EDHF-mediated mechanisms in anandamide-induced relaxation of the rabbit mesenteric artery. Randall and Kendall (1998) demonstrated vasorelaxation of the perfused rat mesenteric arterial bed caused by anandamide, which was sensitive to the combination of apamin and charybdotoxin, supporting the possibility that a component of the anandamide response is EDHF-mediated.

Similarly, the endothelium-dependent vasodilatation induced by anandamide of murine mesenteric arteries was NO and COX-independent but sensitive to the combination of apamin and charybdotoxin (Jarai et al., 1999). The authors concluded that cannabinoid-induced relaxation was mediated by a rimonabant-sensitive endothelial receptor, distinct from the classical cannabinoid receptors, which may be coupled to release of an EDHF (Jarai et al., 1999). However, the effect of rimonabant on vasodilatation in the CB-receptor knockout mice could be as a result of its ability to inhibit gap-junctional communication. Harris et al. (2000) had previously implicated the involvement of gap junctions in EDHF-mediated relaxation of rat mesenteric arterial bed. The same group also linked gap-junctional communication to the vasorelaxation of
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rat mesenteric arterial bed to anandamide, which was attenuated by high concentrations of rimonabant and 18β-glycyrrhetinic acid. However, the response to anandamide was predominately endothelium-independent and involved the activation of sensory nerves, possibly through the release of NO as a neurotransmitter (Harris et al., 2002).

Other studies failed to substantiate the effects of apamin and charybdotoxin on anandamide-induced relaxation of arteries from the rat mesenteric arterial bed. In isolated mesenteric arteries vasorelaxation was sensitive to the inhibition of only large conductance Ca²⁺-activated K⁺-channels (Plane et al., 1997) and was insensitive to the combination of apamin and charybdotoxin (White and Hiley, 1997).

In summary, anandamide has been demonstrated to elicit endothelium dependent vasorelaxation via similar mechanism as EDHF, possibly including the release of an EDHF. Although, experiments using the same tissue type have yielded conflicting results in relation to the involvement of K⁺-channels and gap-junctions.

1.5.6 A novel non-classical cannabinoid receptor

A novel endothelial cannabinoid receptor distinct from both CB₁ and CB₂ receptors, has been implicated in mediating the vasorelaxant effects of cannabinoids. Investigations by Wagner et al. (1999) described endothelium-dependent vasodilation to anandamide in the rat mesenteric arterial bed that was attenuated by high concentrations of rimonabant (1-5µM). Although, the authors demonstrated that other potent cannabinoid-receptor agonists, WIN-55212 and HU-210, failed to elicit vasodilatation. It was postulated that a rimonabant-sensitive non-CB₁/CB₂ receptor present on the endothelium was the target for anandamide (Wagner
et al., 1999). However, it is possible that in the mesenteric vasculature the sensitivity of anandamide responses to rimonabant may be a result of its effects in blocking gap-junctional communication at higher concentrations. Chaytor et al. (1999) demonstrated that rimonabant (10µM) blocked dye transfer in COS-7 cells and mimicked the effects of gap-junction inhibitors in blocking anandamide-induced relaxation of rabbit mesenteric arteries. Gap-junction inhibitors have also been shown to attenuate vasorelaxation of rat mesenteric arterial bed to anandamide (Harris et al., 2002).

Work by Jarai et al. (1999) presented further evidence supporting the existence of a novel endothelial cannabinoid receptor. It was demonstrated that abnormal cannabidiol (abn-cbd), which does not bind to CB₁ receptors, caused vasodilation of mouse mesenteric vasculature that was inhibited by rimonabant (1µM). This was replicated in CB₁ and CB₂ receptor knockout mice (Jarai et al., 1999). It was proposed that the novel cannabinoid receptor was coupled to EDHF-release as abn-cbd-induced vasodilation was blocked by the combination of charybdotoxin and apamin (Jarai et al., 1999). As discussed earlier, this is a well defined characteristic of the EDHF response. The novel cannabinoid receptor has been reported to be inhibited by cannabidiol and O-1918 (Jarai et al., 1999; Offertaler et al., 2003). Subsequently, a number of studies have also implicated the novel cannabinoid receptor in vasorelaxant responses. Vasorelaxation of small resistance mesenteric arteries by anandamide was sensitive to 0-1918 and reduced by the combination of charybdotoxin and apamin (O'Sullivan et al., 2004). Similarly, oleamide-induced relaxation of small mesenteric arteries is also sensitive to 0-1918, rimonabant (3µM) and a combination of charybdotoxin and apamin. Vasorelaxation of rat and rabbit aortae is also partly mediated by a novel endothelial cannabinoid receptor (Herradon et al., 2007; Mukhopadhyay et al., 2002).
Figure 1.2 Summary of the mechanisms involved in vasorelaxation that have been discussed in Chapter 1. Endothelium-dependent agonists, such as acetylcholine and bradykinin, can activate GCRP to upregulate protein kinases. Phospholipase C (PLC) can cause the increase of intracellular calcium (Ca\(^{2+}\)), which activates endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) that can
diffuse to the smooth muscle to stimulate cGMP. Increase in endothelial calcium can also stimulate intermediate and small calcium-activated potassium channels driving the efflux of potassium ions (K\(^{+}\)) and the release of EDHF. Efflux of K\(^{+}\) can activate inwardly rectifying potassium (K\(_{ir}\)) and the sodium-potassium (Na\(^{+}\)-K\(^{+}\)) pump. EDHF may include epoxyeicosatrienoic acids (EETs) that can activate smooth muscle large conductance potassium channels (bK\(_{ca}\)) to hyperpolarise smooth muscle, hydrogen peroxide (H\(_{2}\)O\(_2\)) or C-type natriuretic peptide (CNP) that can activate receptor-mediated increases in smooth muscle cGMP. EDHF may be coupled to a novel endothelial cannabinoid receptor (CBx) and in some cases hyperpolarisation is potentiated via myoendothelial gap junctions. Phospholipase A (PLA) can liberate arachidonic acid from the phospholipid bilayer, which can be metabolised by the cyclooxygenase pathway (COX) to produce prostaglandin (PGI\(_{2}\)). PGI\(_{2}\) can act to increase cAMP levels in smooth muscle cells by activating IP receptors. Activation of TRPV1 receptors on perivascular sensory nerves can release the neuropeptide calcitonin gene related peptide (CGRP) that stimulates the CGRP receptor to increase smooth muscle cAMP. The activation of TRPV1 can also release NO. All these mechanisms result in the relaxation of vascular smooth muscle causing vasorelaxation.

Figure 1.2 has been adapted from Randall et al. (2004), Luksha et al. (2009) and Giles et al. (2012).
1.5 Hypotensive effects of endocannabinoids

Endogenous cannabinoids and phytocannabinoids have received growing interest because of their potential therapeutic use as antihypertensive agents (reviewed; Pacher et al., 2005; Sarzani, 2008; Cunha et al., 2011). It has been postulated that endocannabinoids may contribute to the hypotensive state of certain pathophysiological diseases, including; haemorrhagic shock (Wagner et al., 1997), endotoxic shock (Varga et al., 1998; Wang et al., 2001; Liu et al., 2003; Batkai et al., 2004), cardiogenic shock (Wagner et al., 2001) and liver cirrhosis (Batkai et al., 2001; Ros et al., 2002; Domenicali et al., 2005). Recent research has implicated an upregulated endocannabinoid system, involving increased CB$_1$-receptor expression and endocannabinoid production in liver cirrhosis (Caraceni et al., 2010). Advanced liver cirrhosis is associated with vasodilation of the mesenteric vasculature (Piscaglia et al., 1997), which can contribute to portal hypertension by enhancing portal inflow. Advanced cirrhosis is also associated with systemic hypotension caused by vasodilation (Chu et al., 1997; Van Roey et al., 1997). Research by Batkai et al. (2001) has suggested that the endocannabinoid signalling system may contribute to cirrhotic-induced low blood pressure. The hypotensive state of rats with biliary cirrhosis and CCl$_4$-induced cirrhotic model was normalised by the CB$_1$-receptor antagonist rimonabant (Batkai et al., 2001). Circulating monocytes isolated from biliary cirrhotic rats and human cirrhotic patients, when injected into normal rats elicited a long-lasting hypotension but had no effect in rimonabant-treated rats (Batkai et al., 2001). Monocytes from cirrhotic rats where shown to have enhanced levels of anandamide by 2-3 fold when compared to monocytes from healthy rats. Increased anandamide levels correlated with greater expression of CB$_1$-receptors in endothelial cells from hepatic arteries (Batkai et al., 2001).
The prototypical endocannabinoid, anandamide has been demonstrated to cause hypotension in vivo (Varga et al., 1995; 1996). Intravenously administered anandamide elicits a triphasic blood pressure response, characterised by an initial drop in blood pressure, a subsequent pressor response, followed by a sustained hypotension (Varga et al., 1995; 1996). The prolonged hypotension is associated with reduced total peripheral resistance and reduced cardiac contractility and is absent in CB$_1$-receptor knockout mice (Ledent et al., 1999) and in the presence of rimonabant (Lake et al., 1997; Malinowska et al., 2001). However, the triphasic response to anandamide including the prolonged phase of hypotension is sensitive to the type of in vivo model used. Prolonged hypotension in response to anandamide is either completely absent or greatly attenuated in conscious models (Lake et al., 1997; Gardiner et al., 2002). Gardiner et al. (2002) described complex pressor effects in conscious rats, where transient pressor responses were characterised by vasoconstriction in regional vascular beds, while increasing doses of anandamide induced transient hypotension followed by a prolonged period of hypertension. In contrast, a biphasic response characterised by an initial transient drop in blood pressure followed by prolonged hypotension has been reported in conscious mice models (Ledent et al., 1999). Conscious CB$_1$-receptor knockout mice failed to respond to anandamide in the characteristic biphasic manner, suggesting the hypotensive effects of anandamide were mediated by cannabinoid receptors (Ledent et al., 1999).

Smith and McQueen (2001) reported a dose-dependent drop in blood pressure to anandamide administered to the vasculature of the hindlimb of the anesthetised rat, results which were mimicked by the vanilloid capsaicin. The blood pressure response evoked by both anandamide and capsaicin was attenuated by capsazepine, a competitive TRPV1 antagonist (Smith and McQueen, 2001). This
suggests that cardiovascular responses to endocannabinoids can be mediated by perivascular TRPV1 containing sensory nerves. This is consistent with the observations of Zygmunt et al. (1999) who showed capsaicin-sensitive sensory nerves to be the driving mechanism behind anandamide-induced vasorelaxation of rat isolated arteries, via the release of the calcitonin gene related peptide. In TRPV1 knockout mice the long lasting hypotensive phase in response to anandamide remained intact, but the initial transient drop in blood pressure was decreased.

1.6 Aims

The primary aim of this study was to examine the vascular actions of the endocannabinoid-like mediator oleamide in comparison with the more widely studied endocannabinoid, anandamide. It is hypothesised that oleamide will demonstrate a similar vasorelaxant profile to anandamide. To fulfil our aims several different methods were used. Initially, the effects of oleamide in larger conduit vessels, namely, the rat isolated aorta, porcine isolated coronary and mesenteric arteries were examined as, to date, the actions of oleamide have only been characterised in small resistance vessels. Although a large body of research has characterised the cardiovascular effects of anandamide in a number of models of hypertension and implicated the endocannabinoid system in hypertension, no investigation to date has described the responses to oleamide in a model of hypertension. To achieve this, I compared the vascular actions of oleamide and anandamide in arteries from the spontaneously hypertensive rat model in comparison to the normotensive control, the Wistar-Kyoto. As a consequence of studies into the mechanisms driving vasorelaxation, the vascular actions of the TRPV1 agonist capsaicin was subsequently characterised in greater detail. The present study also
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characterised the vasodilator properties of oleamide in a whole arterial bed preparation, the rat mesenteric arterial bed.
Chapter 2

The vascular actions of oleamide in conduit arteries
Chapter 2 The vascular actions of oleamide in conduit arteries

2.1 Introduction

Oleamide (cis-9, 10-octadecenoamide) is a fatty acid primary amide that was originally isolated from the cerebrospinal fluid of cats subjected to sleep deprivation (Cravatt et al., 1995). Being a fatty acid amide, oleamide shares structural similarity with the prototypical endocannabinoid anandamide, thus both act as a substrate for fatty acid hydrolase (FAAH). Oleamide administered in vivo demonstrates a similar profile of effects to anandamide. Indeed, oleamide induces the tetrad of behavioural effects used to denote cannabinoid-like activity. Oleamide causes hypomotility (Basile et al., 1999; Federova et al., 2001), analgesia (Federova et al., 2001), and hypothermia (Federova et al., 2001; Huitron-Resendiz et al., 2001) in rat and mice models. A number of the in vivo effects elicited by oleamide are sensitive to a blockade of CB$_1$ receptors, for example, the analgesic effect of oleamide in the tail-flick assay was abolished by rimonabant (Fedorova et al., 2001). Oleamide also increases appetite (Huitron-Resendiz et al., 2001), which is a classic cannabinoid-induced effect reflected in the success of rimonabant in reducing body weight (Leite et al., 2006). In addition oleamide has been shown to be an endogenous agonist of both rat and human CB$_1$ receptors (Legett et al., 2004).

Oleamide has recently been described as a potent vasodilator of rat mesenteric resistance arteries. Hoi and Hiley (2006) reported that oleamide-induced vasorelaxation involved sensory nerve-mediated activity, activation of calcium activated K$^+$-channels and was partly dependent on an intact endothelium. Oleamide-induced responses were also sensitive to O-1918, an antagonist of the putative endothelial CB receptor (Hoi and Hiley, 2006). Sudhahar et al. (2009) also implicated non-endothelial TRPV1 receptors and an O-
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1918 sensitive component, as well as endothelial CB₁ receptors. The literature describes a clear overlap of mechanisms involved in responses to oleamide and anandamide.

While a large number of studies have investigated the vascular effects of anandamide, oleamide has received little attention. With this in mind the principal aim of this chapter was to characterise and compare the vascular responses to oleamide and anandamide in larger conduit arteries. The responses to oleamide in rat aortae, porcine coronary arteries and porcine mesenteric arteries were investigated.
2.2 Methods and Materials

2.2.1 Animals

Wistar rats (250-350g; aged 12-18 weeks) were used during this investigation. All rats used were housed at the Biomedical Services Unit, University of Nottingham with a 12h light/dark cycle and in temperature-controlled conditions. Porcine hearts and porcine mesenteries were obtained from a local abattoir and transported in ice-cold modified Krebs’-Henseleit buffer solution (NaCl 118, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 4.2, NaHCO$_3$ 25, D-glucose 10, CaCl$_2$ 2 (mM)).

2.2.2 Preparation of aortic rings and experimental protocol

The rats were stunned by a blow to the back of the head and killed by cervical dislocation. This was followed by the removal of the abdominal aorta. Subsequently, the aorta was cleared of all connective tissue by blunt dissection and cut into segmental rings (3mm-5mm). The aortic rings were placed between two metal wires, with one being fixed and the other attached via thread to an isometric transducer and placed into 50ml organ baths (Figure 2.1). The organ baths were filled with modified Krebs’-Henseleit buffer solution (NaCl 118, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 4.2, NaHCO$_3$ 25, D-glucose 10, CaCl$_2$ 2 (mM)) at 37°C and gassed (5% CO$_2$/95% O$_2$). The aortic rings were allowed to equilibrate to an optimal baseline tension of 9.8mN for 1h. Tension was measured by a Leitica force transducer coupled to an ADInstruments MacLab recording system.
Figure 2.1 Isometric tension set up. A=pressure transducer, B=thread, C=glass rod, D=gas inlet, E=connection to thermocirculator, F=modified Kreb’s solution (50ml), G=organ bath, H=organ bath drainage outlet, I=upper mounting hook, J=lower mounting hook, K=gas delivery tube, L=tissue segment and M=heated chamber.
Vessels were pre-contracted with the $\alpha_1$-adrenoceptor agonist methoxamine (1-10µM) to achieve submaximal contraction prior to the addition of oleamide (10nM-10µM) (Figure 2.2) or anandamide (1nM-10µM), which were added cumulatively at 5 minute intervals to construct concentration-response curves. Pre-contraction data can be found in the appendices. Concentration-response curves were started after a stable pre-contraction was established (after approx 20 minutes). Pre-contraction data can be found in the results section.

Figure 2.2

![Graph showing concentration-response curves](image)

Figure 2.2 Representative trace of the response to oleamide in the rat isolated aorta. A= 10nM, B=30nM, C=100nM, D=300nM, E=1µM, F=3µM and G=10µM.

To investigate the role of sensory nerves, some vessels were subjected to pre-treatment with 10µM capsaicin for 1h (Zygmunt et al., 1999). Capsaicin pre-treatment was followed by a 20min wash-out prior to methoxamine contraction. Experiments were also performed in the presence of capsazepine (5µM), a TRPV$_1$ receptor antagonist (White et al., 2001). To further analyse sensory nerve activity of aortae, concentration-response curves to capsaicin (1nM-10µM) were constructed cumulatively. Experiments were also
carried out in the presence of ruthenium red (10µM), a blocker of capsaicin-activated cation channels (Harris et al., 2002).

The contribution of the endothelium was assessed by denuding some vessels of endothelial cells. This was achieved by gentle rubbing of the intimal surface with metal forceps. Endothelial function was examined by addition of carbachol (10µM) after pre-contraction by methoxamine. Vessels responding with less than 20% relaxation of induced tone to carbachol were deemed to be lacking a functional endothelium. In some vessels, incubated with L-NAME (300µM) to inhibit nitric oxide synthase, both control (n=7) and treated (n=7) arteries were unresponsive to oleamide. In other vessels indomethacin (10µM) was present to inhibit cyclooxygenase (O'Sullivan et al., 2004). Some experiments were carried out in the presence of high extracellular K⁺ (NaCl 62.5, KCl 59.4, MgSO₄ 1.2, KH₄PO 1.2, NaHCO₃ 25, D-glucose 10, CaCl₂ 2 (mM)) to assess the role of hyperpolarising mechanisms in responses to oleamide. In some experiments the role of cannabinoid CB₁ receptors was examined by incubation with AM251 (1µM) a cannabinoid CB₁ receptor antagonist (O'Sullivan et al., 2005). Some experiments were carried out in the presence of URB597 (1µM), a fatty acid amide hydrolase (FAAH) inhibitor (Ho and Randall, 2007).

2.2.3 Preparation of porcine coronary arteries and mesenteric arteries and experimental protocol

Proximal coronary arteries were removed from the hearts and first order mesenteric arteries removed from mesenteries and stored in a refrigerator over-night at 4°C in pre-gassed Krebs’-Henseleit buffer solution having been cleaned thoroughly of connective tissue by blunt dissection and cut into segments of 3-5mm. Porcine arteries were stored in the refrigerator no longer than 24h.
Porcine coronary and mesenteric arteries were placed onto two metal wires, with one being fixed and the other attached via thread to an isometric transducer measuring tension and placed into 50ml organ baths (Figure 2.1). The organ baths were filled with modified Krebs’-Henseleit buffer solution at 37°C and gassed steadily (5% CO₂/95% O₂). Porcine coronary arteries and mesenteric arteries were equilibrated to 49mN. Tension was measured by a Leitica force transducer coupled to an ADInstruments MacLab recording system. Porcine coronary and mesenteric arteries were pre-contracted with U46619 (1-70nM), a thromboxane mimetic, prior to the construction of concentration-response curves to oleamide (10nM-10µM) and anandamide (1nM-10µM) (Figure 2.3) to achieve a submaximal contraction of 50-80% of maximal KCl (60mM) response. To investigate the role of sensory nerves in the response to anandamide, porcine mesenteric arteries were subjected to pre-treatment with 10µM capsaicin for 1h (Zygmunt et al., 1999). Capsaicin pre-treatment was followed by a 20min wash-out period, prior to pre-contraction. In porcine mesenteric arteries, concentration-responses curves to anandamide were also constructed in the presence L-NAME (300µM), indomethacin (10µM) and in endothelial denuded vessels.

Figure 2.3
Figure 2.3 Representative trace of the response to anandamide in a porcine mesenteric arterial segment. A=1nM, B=3nM, C=10nM, D=30nM, E=100nM, F=300nM, G=1µM, H=3µM and I=10µM.

### 2.2.4 Drugs and reagents

Oleamide (*cis*-9-Octadecenoamide), anandamide (arachidonylethanolamide) and AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) were purchased from Tocris Co. (UK). Capsaicin (8-Methyl-N-vanillyl-*trans*-6-nonenamide), capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), methoxamine hydrochloride (α-(1-Aminoethyl)-2,5-dimethoxybenzyl alcohol hydrochloride), U46619 (9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F$_{20}$), ruthenium red (ammoniated ruthenium oxychloride), L-NAME (NG-Nitro-L-arginine methyl ester hydrochloride), indomethacin (1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid), URB597 (cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester) and carbachol ((2-Hydroxyethyl) trimethylammonium chloride carbamate) were all purchased from Sigma Chemicals Co. (UK). Anandamide, capsaicin, indomethacin and capsazepine were dissolved in ethanol at stock concentrations of 10 mM and methoxamine at 1mM. DMSO (Dimethyl sulfoxide) was used to dissolve stock solutions of oleamide and AM251 at 10 mM and URB597 at 1 mM. All other drugs were dissolved using distilled water. All dilutions were made using distilled water. L-NAME, indomethacin, AM251 and capsazepine were incubated for approximately 20 minutes before pre-contraction of vessels with methoxamine. Ruthenium red was incubated for 30 minutes, while URB597 was incubated for 10 minutes before pre-contraction.
2.2.5 **Statistical analysis**

All responses are expressed as mean percentage vasorelaxation with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0 software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(\text{LogEC}_{50} - X) \times \text{Hillslope})}}, \]

when \( X = \) logarithm of agonist concentration and \( Y = \) response from Bottom to Top in a sigmoidal shape. The curves were used to determine potency (pEC\(_{50}\)) and maximal response (R\(_{\text{max}}\)) values. Potency (pEC\(_{50}\)) is the negative log of agonist concentration that reduced methoxamine-induced contraction by 50%. The maximal response relates to the maximum percentage vasorelaxation of methoxamine-induced pre-contraction. Statistical significance was determined using two-tailed unpaired Student’s t-test between two data sets or one-way ANOVA when comparing multiple data sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. Statistical significance was determined using one-way ANOVA unless stated otherwise in the figure legend. P-values <0.05 were considered significant.

2.2.6 **Isolation and culture of DRG neurones**

Dorsal root ganglion (DRG) neurons are used as a model for primary afferent nociceptors and have a high expression of TRPV1 receptors. DRG cells were therefore used as a tool to investigate the effects of oleamide on TRPV1 receptors. The following protocol was carried out by Paul Millns of the University of Nottingham. DRG neurons were isolated from Wistar rats (250g-350g) (Lindsay, 1988) and after the rats were killed as previously described.

After being killed as previously described the rat was sprayed with 70% alcohol. The skin was cut to expose the underlying muscle and vertebral column. Incisions were made either side of the vertebral
column and spinous processes were then removed from the dorsal surface of the exposed spine. After this cleaning of the spinal column it was removed from the animal. Muscle and connective tissue was then cleaned from the vertebral column before it was placed in Hank’s balanced saline solution (HBSS) containing HEPES (20mM). Using fine-pointed scissors a ventral strip of bone (3-4mm) was removed from the ventral roof of the vertebral column. An incision to the mid-line of the spinous processes allowed the bisection of the spinal column. Following the gentle removal of the spinal cord the DRGs were exposed. Approximately 30-40 DRGs were cut from the central and peripheral trunks using microsurgery scissors and watch-makers forceps and placed in HBSS with HEPES (0.1M) and washed once.

Ganglia were cultured in agreement to the methodology outlined in Millns et al. (2006). DRG neurons were incubated (37°C/5% CO₂) for 90min in 5mL neurobasal medium (Invitrogen, Paisley, UK) containing collagenase (2.5mg/mL) and 10% horse serum. Isolated ganglia were washed in phosphate-buffered saline (5mL) with porcine trypsin (2.5mg/mL) and incubated for 30 min (37°C/5% CO₂). Thereafter, ganglia were washed and triturated. The subsequent cell suspension was layered on a 16% bovine serum albumin solution (4mL), which was centrifuged allowing the removal of the supernatant. The remaining cell sediment was suspended again in neurobasal medium (1mL) and made up to 1.5mL with glial cell line-derived neurotrophic factor (50ng/mL), nerve growth factor (25pg/mL), L-glutamate (2mM), penicillin (200 units/mL), streptomycin (200ng/mL) and with horse serum. Cell suspension was pipetted onto 13mm glass cover slips and incubated (37°C/5% CO₂) overnight.
Fura-2AM is a calcium sensitive dye and loading cells with Fura-2AM allows intracellular Ca^{2+} concentrations to be visualised using imaging techniques as the excitation wavelength of Fura-2AM alters upon binding of calcium. After being grown overnight DRG cells were washed three times with a Ca^{2+} buffer and a 2.5µL Fura-2AM solution (5µM) in 450µL Ca^{2+} buffer (NaCl 145, KCl 5, CaCl_2 2, MgSO_4·7H_2O 1, HEPES 10, glucose 10 (mM)) with 50µL horse serum and then incubated (37°C) in the dark. After at least 30mins DRG cells were again washed three times with a Ca^{2+} buffer to remove excess Fura-2AM and left for 15mins. The intracellular calcium concentrations of 30-40 cells from individual neurones were analysed using an Improvision imaging system. The n numbers used in the results section represent the number of cells used. Cells were isolated from six different animals. Intracellular calcium was estimated as the ratio of fluorescence intensities emitted at 340nm and 380nm excitation wavelengths (measured at 500nm). Cells were imaged using an inverted microscope with a high sensitivity camera attached. Excitation wavelengths were altered by a filter wheel attached to the microscope. Cover slips with DRG cells were attached to a Perspex chamber using vacuum grease. This formed a well that was then attached to a heated platform (30°C) and DRG neurons were perfused (2ml/min) with Ca^{2+}-buffer. Cover slips containing DRG cells were then superfused (2ml/min) with capsaicin (100nM) and oleamide (100µM) for 60s. Responses to oleamide and capsaicin alone were expressed as a percentage of the response to high KCl (60mM)(60s) and data are expressed as mean ± standard error of the mean (SEM). Perfusion of DRGs with KCl results in calcium-influx caused by depolarisation and was used as a control response during the experiments. DRGs that demonstrated an increase from basal in the ratio of peak fluorescence that was less
than 0.2 in response to KCl were excluded from the study for being non-viable.

2.3 Results

2.3.1 Vascular responses to oleamide in the rat isolated aorta

Oleamide caused concentration-dependent vasorelaxation of aortic rings isolated from Wistar rats ($R_{\text{max}}=20.7\pm2.5\%, \ pEC_{50}=6.64\pm0.37, \ n=10$) (Figure 2.4). DMSO (0.15% of final bath volume) did not cause a vascular response.

Figure 2.4 Vasorelaxant responses to oleamide and vehicle control (DMSO) in rat aortic rings pre-contracted with methoxamine. Mean data with bars indicating S.E.M are displayed.
2.3.2 Vascular responses to oleamide in the rat isolated aorta in the presence of high extracellular $K^+$

Vasorelaxant responses to oleamide ($R_{\text{max}}=16.9\pm2.2\%$, $\text{pEC}_{50}=7.27\pm0.54$, $n=5$) in rat aortae were unaffected by a high concentration of extracellular $K^+$ (60mM) ($R_{\text{max}}=15.9\pm2.1\%$, $\text{pEC}_{50}=6.73\pm0.45$, $n=8$) (Figure 2.5).

Figure 2.5

Figure 2.5 Vasorelaxant responses to oleamide in aortic rings from Wistar rats contracted with high extracellular $K^+$ (60mM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.3 Vascular responses to oleamide in the rat isolated aorta after a 1h capsaicin pre-treatment

Oleamide caused vasorelaxant responses in aortae such that, at 10µM, relaxation was 14.0±2.8% (n=11) and this response was abolished after pre-treatment of vessels with capsaicin (n=11) (Figure 2.6).

Figure 2.6 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-treated with capsaicin (10µM). Mean data with bars indicating S.E.M are displayed.
2.3.4 Vascular responses to oleamide in the rat isolated aorta in the presence of vanilloid receptor antagonists

Vasorelaxant responses to oleamide in rat aortic rings ($R_{\text{max}}=20.7\pm2.5\%$, $pEC_{50}=6.64\pm0.37$, $n=10$) were unaffected by the TRPV1 antagonist capsazepine (5µM) ($R_{\text{max}}=26.7\pm4.0\%$, $pEC_{50}=6.80\pm0.51$, $n=7$) (Figure 2.7). Similarly, oleamide-induced vasorelaxation ($R_{\text{max}}=21.9\pm3.6\%$, $pEC_{50}=6.03\pm0.35$, $n=7$) was unaffected by the cation channel blocker ruthenium red (10µM) ($R_{\text{max}}=24.1\pm4.8\%$, $pEC_{50}=5.66\pm0.33$, $n=7$) (Figure 2.8).

Figure 2.7

Figure 2.7 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-contracted with methoxamine in the presence of capsazepine (5µM). Mean data with bars indicating S.E.M are displayed.
Figure 2.8 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-contracted with methoxamine in the presence of ruthenium red (10µM). Mean data with bars indicating S.E.M are displayed.
2.3.5 Vascular responses to oleamide in the rat isolated aorta in the presence of COX-inhibition

Vasorelaxation induced by oleamide in rat aortic rings ($R_{\text{max}}=19.7\pm4.6\%, \ pEC_{50}=5.85\pm0.44, \ n=8$) was unaffected by indomethacin (10µM) ($R_{\text{max}}=31.0\pm9.6\%, \ pEC_{50}=5.30\pm0.34, \ n=8$) (Figure 2.9)

Figure 2.9

Figure 2.9 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-contracted with methoxamine in the presence of indomethacin (10µM). Mean data with bars indicating S.E.M are displayed.
2.3.6 Vascular responses to oleamide in endothelium denuded aortic rings

Vasorelaxation elicited by oleamide in rat aortic rings ($R_{\text{max}}=14.5\pm6.0\%$, $\text{pEC}_{50}=6.04\pm1.02$, $n=6$) was comparable to the vasorelaxation of endothelial denuded vessels ($R_{\text{max}}=13.8\pm1.5\%$, $\text{pEC}_{50}=6.32\pm0.25$, $n=6$) (Figure 2.10).

Figure 2.10

![Graph showing vasorelaxant responses to oleamide in endothelium denuded aortic rings from Wistar rats pre-contracted with methoxamine. Mean data with bars indicating S.E.M are displayed.](image-url)
2.3.7 Vascular responses to oleamide in the presence of URB597 in the rat isolated aorta

Vasorelaxation induced by oleamide in rat aortic rings ($R_{\text{max}}=10.2\pm3.5\%$, $p\text{EC}_{50}=5.88\pm0.50$, $n=7$) was unaffected by the presence of URB597 (1µM) ($R_{\text{max}}=13.0\pm2.0\%$, $p\text{EC}_{50}=6.36\pm0.28$, $n=8$) (Figure 2.11).

Figure 2.11 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-contracted with methoxamine in the presence of URB597 (1µM). Mean data with bars indicating S.E.M are displayed.
2.3.8 Vascular responses to oleamide in the presence of AM251 in the rat isolated aorta

Vasorelaxation induced by oleamide in rat aortic rings ($R_{\text{max}}=10.5\pm4.4\%$, $pEC_{50}=5.61\pm0.43$, $n=7$) was unaffected by the presence of AM251 (1µM) ($R_{\text{max}}=11.6\pm3.4\%$, $pEC_{50}=5.75\pm0.48$, $n=6$) (Figure 2.12).

Figure 2.12

Figure 2.12 Vasorelaxant responses to oleamide in aortic rings from Wistar rats pre-contracted with methoxamine in the presence of AM251 (1µM). Mean data with bars indicating S.E.M are displayed.
2.3.9 Vascular responses to anandamide in the rat isolated aorta

Anandamide (1nM-10µM) failed to elicit any response in rat isolated aortic rings (n=8) (Figure 2.13).

Figure 2.13 Vascular responses to anandamide in aortic rings from Wistar rats pre-contracted with methoxamine. Mean data with bars indicating S.E.M are displayed.
2.3.10 Vascular responses to oleamide in porcine coronary arteries

Oleamide (10nM-30µM) and DMSO (0.15% of final bath volume) failed to elicit any response in isolated porcine coronary arteries (n=6) (Figure 2.14).

Figure 2.14

Figure 2.14 Vascular responses to oleamide (10nM-30µM) and vehicle control (DMSO) in porcine coronary arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed.
2.3.11 Vascular responses to anandamide in porcine coronary arteries

Anandamide failed to elicit a vascular response in porcine isolated coronary arteries (n=5) (Figure 2.15).

Figure 2.15 Vascular responses to anandamide in porcine coronary arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed.
2.3.12 Vascular responses to oleamide in porcine isolated mesenteric arteries

Oleamide did not cause a significant vasorelaxant response in porcine isolated mesenteric arteries compared to DMSO (0.15% of final bath volume) (Figure 2.16).

Figure 2.16

Figure 2.16 Vascular responses to oleamide (10nM-10µM) and vehicle control (DMSO) in porcine mesenteric arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.13 Vascular responses to anandamide in porcine isolated mesenteric arteries

Anandamide and capsaicin caused concentration-dependent vasorelaxation of porcine mesenteric arteries, such that at 10µM, anandamide elicited relaxation of 53.1±13.4% (n=6) (Figure 2.17). At the same concentration the vehicle control (0.15% of final bath volume) did not cause vasorelaxation.

Figure 2.17

![Graph showing vasorelaxant responses to anandamide and vehicle control](image)

Figure 2.17 Vasorelaxant responses to anandamide and vehicle control (ethanol) in porcine mesenteric arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed.
2.3.14 Vascular responses to anandamide in the presence of L-NAME in porcine isolated mesenteric arteries

The vasorelaxant response to 10µM anandamide, 34.2±8.3% (n=8) in porcine mesenteric arteries was abolished in the presence of L-NAME (300µM) (Figure 2.18). Arteries in the presence of L-NAME were noticeably more sensitive to U46619, with a range of 1-10nM always sufficient to pre-contract vessels.

Figure 2.18

Figure 2.18 Vasorelaxant responses to anandamide in porcine mesenteric arteries pre-contracted with U46619 in the presence of L-NAME (300µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.15 Vascular responses to anandamide in the presence of indomethacin in porcine isolated mesenteric arteries

The vasorelaxant response to 10 µM anandamide, was 31.6±8.5% (n=10), was not significantly affected by indomethacin, 16.0±6.1% (n=10) (Figure 2.19).

Figure 2.19 Vasorelaxant responses to anandamide in porcine mesenteric arteries pre-contracted with U46619 in the presence of indomethacin (10µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.16 Vascular responses to anandamide after a capsaicin pre-treatment in porcine isolated mesenteric arteries

The vasorelaxant response to anandamide, 23.0±5.3% (n=6), was unaffected by a 1h pre-treatment of vessels with capsaicin, 15.4±7.4%, (n=6) (Figure 2.20).

Figure 2.20

![Figure 2.20](image-url)

Figure 2.20 Vasorelaxant responses to anandamide in porcine mesenteric arteries pre-contracted with U46619 after a 1h capsaicin pre-treatment (10µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.17 Vascular responses to anandamide in endothelium-denuded porcine mesenteric arteries

Vasorelaxation of porcine mesenteric arteries by 10µM anandamide, 17.5±5.8% (n=7), remained similar in endothelium denuded vessels, such that vasorelaxation by 10µM was 13.1±3.6% (n=8) (Figure 2.21).

Figure 2.21

Figure 2.21 Vasorelaxant responses to anandamide in endothelial denuded porcine mesenteric arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
2.3.18 Effects of capsaicin and oleamide on calcium influx in rat dorsal root ganglion neurones

Capsaicin produced a marked increase in intracellular calcium that was 121±5. % (n=244) of the response elicited by KCl. Conversely, oleamide produced a small increase in intracellular calcium, which was 10.3±6.6% (n=244) of the KCl response (Figure 2.22).

Figure 2.22

![Graph showing intracellular calcium increases in Wistar rat DRG neurones in response to capsaicin (100nM) and oleamide (100µM) expressed as a percentage of the response to KCl (60mM).]
2.4 Discussion

The primary aim of this chapter was to characterise the vascular effects of the endocannabinoid substance oleamide and make a comparison to those of anandamide in large conduit arteries. A major finding of the current study is that oleamide demonstrated vasorelaxant properties in the rat isolated aorta, eliciting approximately 20% relaxation. However, oleamide failed to cause any vascular response in porcine mesenteric and coronary arteries.

The magnitude of the vasorelaxant response to oleamide in the rat aorta is similar to the responses reported elsewhere to other cannabinoids. O’Sullivan et al. (2005) described relaxations to anandamide, Δ⁹-tetrahydrocannabinol (THC) and N-arachidonoyldopamine (NADA), all of which caused approximately 20% relaxation of aortic rings. However, the present study demonstrated that anandamide did not cause vasorelaxation of aortae. This is also in contrast to Herradon et al. (2007), who reported a 35% relaxation to anandamide (10nM-100µM) compared to vehicle. Possible explanations for this discrepancy are that the lower concentration of anandamide examined here compared to O’Sullivan et al. (2005) (10µM vs. 30µM) and using ethanol as a solvent may blunt anandamide-induced responses (Herradon et al., 2007).

The present chapter has described a small vasorelaxant effect in rat aortae to oleamide of approximately 20%, which is a considerably reduced response to oleamide compared to previously reported effects in small resistance vessels. Hoi and Hiley (2006) reported approximately 100% relaxation of pre-constricted small mesenteric arteries, while Sudhahar et al. (2009) described a 60% vasorelaxant response to oleamide. It is therefore possible that small resistance vessels are more sensitive to the effects of oleamide than larger conduit vessels. This is further demonstrated by the lack of a
response to oleamide in porcine coronary arteries. Indeed, differences in the reactivity to anandamide exist between conduit and resistance arteries (O’Sullivan et al., 2004). O’Sullivan et al. (2004) demonstrated that in small resistance arteries anandamide elicited greater vasorelaxation compared to conduit mesenteric arteries. Anandamide caused 90% relaxation of pre-constricted third order mesenteric arteries compared to 35% of the superior mesenteric artery. The authors also reported differences between conduit and resistance vessels in the underlying mechanisms involved in vasorelaxation. Anandamide-induced vasorelaxation of large mesenteric arteries was predominately mediated by non-endothelial vanilloid and CB$_1$ receptors, while in smaller mesenteric arteries responses involved an EDHF component (O’Sullivan et al., 2004). This chapter presents results that similarly demonstrate oleamide and anandamide have limited vasodilator actions in larger conduit vessels.

Anandamide can induce vasorelaxation through the activation of TRPV1 receptors in rat hepatic and mesenteric arteries and in guinea pig basilar arteries (Zygmunt et al., 1999). Activation of TRPV1 receptors on perivascular sensory nerves elicits the release of various neuropeptides, including CGRP, to cause vasorelaxation. Indeed, anandamide-induced relaxation of rabbit aortae is partly dependent on non-endothelial TRPV1 activation, a response that is also sensitive to the CGRP antagonist CGRP8-37 (Mukhopadhyay et al., 2002). Past studies have also implicated sensory nerve-mediated mechanisms in oleamide-induced vasorelaxation (Hoi and Hiley, 2006; Sudhahar et al., 2009). With this in mind it was hypothesised that relaxation of rat aortae was mediated by sensory nerves or TRP channels. Indeed, in the rat aorta oleamide-induced vasorelaxation was sensitive to capsaicin pre-treatment designed to desensitise perivascular sensory nerves. In Chapter 3, augmented responses to oleamide and anandamide in the isolated aorta from
the spontaneously hypertensive rat (SHR) model of hypertension were normalised by capsaicin pre-treatment. Previous reports have implicated sensory-nerve mediated mechanisms in regulating the enhanced responses to endocannabinoids in hypertensive models (Li et al., 2003; Wang et al., 2005; Wheal and Randall, 2009).

The sensitivity of oleamide-induced responses to capsaicin pre-treatment suggests the involvement of perivascular sensory nerves and the release of neuropeptides. However, when using additional techniques to elucidate the involvement of TRPV1, including the competitive antagonist capsazepine and the cation channel blocker ruthenium red, oleamide-induced responses remained intact. This clearly shows that vasorelaxation to oleamide is occurring independently of TRPV1 activation but is sensitive to capsaicin. The results also are consistent with an alternative mode of action for the vascular effects of capsaicin. Indeed, Chapter 6 provides evidence that capsaicin can inhibit the influx of calcium through L-type calcium channels.

There is a growing body of evidence that demonstrates that capsaicin can also regulate smooth muscle function by TRPV1 independent mechanisms. Several studies have reported that capsaicin can inhibit calcium channels or activate potassium channels in order to facilitate relaxation (Lo et al., 1995; Ellis et al., 1997; Zhu et al., 1997; Sim et al., 2001; Yeon et al., 2001). Sim et al. (2001) reported that capsaicin dose-dependently inhibited spontaneous contractions in antral circular myocytes of the guinea pig stomach. It was proposed that capsaicin inhibited this spontaneous contraction by direct intracellular inhibition of voltage-operated Ca\(^{2+}\) channels (Sim et al., 2001). In smooth muscle cells of the rat aorta the inhibition of voltage-dependent L-type Ca\(^{2+}\) channels was reported to be the main mechanism driving capsaicin-induced relaxation (Lo et al, 1995). Yeon et al. (2001) described a
capsaicin-induced relaxation of the rabbit coronary artery, which was proposed to be due to the activation of the delayed rectifier K+ channel. In human bronchi smooth muscle and equine tracheal smooth muscle capsaicin-induced relaxation was mediated by charybdotoxin-sensitive large conductance Ca2+ activated K+ channels (Ellis et al., 1997; Zhu et al., 1997). Gupta et al. (2007) reported that capsaicin-induced relaxations in isolated human and porcine arteries were not facilitated via CGRP or Neurokinin1 receptors, indicating responses were independent of neuropeptides release. In addition, responses to capsaicin were independent of NO, vanilloid receptors, voltage-sensitive calcium channels, K+ channels or cAMP mediated mechanisms. Based on this the authors concluded that non-specific mechanisms were involved in capsaicin responses. In the present chapter, it should not be ruled out that capsaicin may persist after the wash-out period and may block additional sites. For example, it is possible that capsaicin could block a novel cannabinoid receptor which mediates oleamide-induced vasorelaxation.

The present chapter also investigated the effects of oleamide on calcium influx in DRG neurones. Capsaicin (100nM) was shown to evoke large increases in [Ca2+]. The increase in [Ca2+] is mediated by TRPV1 receptor activation (Caterina et al., 1997). Anandamide has previously been demonstrated to enhance calcium influx in HEK293 cells expressing recombinant TRPV1 and in rat DRG neurones in a capsazepine sensitive manner (Smart et al., 2000). Similarly, the anandamide analogues methanandamide and palmitoylethanolamide also facilitated calcium influx (Smart et al., 2000). As discussed above TRPV1 has also been implicated in the vascular actions of oleamide (Hoi and Hiley, 2006; Sudhahar et al., 2009). Thus, experiments were designed to examine whether oleamide mimicked the effects of capsaicin at TRPV1. Oleamide (100µM), unlike capsaicin, did not cause a marked increase in
intracellular calcium and this would suggest that it is not a potent activator of TRPV1. This strengthens the conclusion that in the rat aortae oleamide is not causing relaxation through TRPV1 receptors. Oleamide (100µM) caused a small decrease in capsaicin-induced calcium influx. Previous studies have demonstrated cannabinoid mediated inhibition of calcium influx elicited by capsaicin in rat DRG neurones (Millns et al., 2001; Sagar et al., 2005). Intracellular increases of calcium in response to capsaicin were attenuated by HU210 and this inhibitory action was abolished by the CB₁ antagonist rimonabant (Millns et al., 2001). The cannabinoid arachidonyl-2-2choroethylamide also inhibited capsaicin-induced increases in $[Ca^{2+}]_i$ in DRG neurones from sham-operated and neuropathic Sprague-Dawley rats and was similarly sensitive to rimonabant (Sagar et al., 2005). Therefore, it is possible that at concentrations used in DRG neurones oleamide is inhibiting responses to capsaicin, possibly via CB₁ receptors.

Oleamide is a selective agonist of both rat and human CB₁ receptors (Leggett et al., 2004). Leggett et al. (2004) demonstrated that oleamide could competitively inhibit binding of CB₁ agonists and antagonists and also upregulate the binding of $[^3S]$GTPγS to rat brain regions. Oleamide also inhibited forskolin-stimulated cAMP accumulation in mouse neuroblastoma cells (Leggett et al., 2004). A range of behavioural effects induced by oleamide are sensitive to CB₁ antagonism (Federova et al., 2001). Also, CB₁-receptor activation has been implicated in oleamide-induced vasorelaxation of mesenteric resistance arteries (Sudhahar et al., 2009). Sudhahar et al. (2009) demonstrated that the presence of the CB₁ antagonist AM251 caused a rightward shift in oleamide-induced relaxation. Therefore, it was an obvious step to investigate the involvement of CB₁ receptors in the vascular responses to oleamide in rat aortae. However, in the present study AM251 had no effect on the small vasorelaxant effect caused by oleamide. In past studies examining
the vasorelaxant effects of anandamide in the rat aorta, responses were independent of CB$_1$ receptor activation (O'Sullivan et al., 2005; Herradon et al., 2007). Therefore, it is unlikely that oleamide is mediating its vascular effects in rat aorta through CB$_1$. Moreover, vasorelaxant responses to oleamide in isolated aorta from SHR and and the normotensive Wistar Kyoto (WKY) control described in Chapter 3 were insensitive to AM251 presence.

As detailed in the methods, in both the control vessels and those treated with L-NAME oleamide failed to elicit a vasorelaxant response in the Wistar aorta. The disappearance of the vasorelaxant effect of oleamide is not without precedent. Previously, Hoi and Hiley (2006) found a large variability in the sensitivity of mesenteric arteries to oleamide from rat to rat. The authors reported that in approximately 30% of the rats used oleamide elicited less than 20% vasorelaxation of pre-constricted tone, compared to the 100% relaxation in vessels from responsive rats and unresponsive vessels were removed from the analysis. Interestingly, mesenteric arteries classed as low-responding to oleamide also responded poorly to anandamide in comparison to mesenteric arteries in which oleamide was efficacious (Hoi and Hiley, 2006). This supports our findings that there is variability in the responses to oleamide, with some aortae being completely unresponsive. The reasons for this are unknown but may reflect seasonal or underlying genetic differences.

The involvement of the COX-pathway in regulating the vascular responses to oleamide in WKY aortae has been discussed elsewhere (Hopps et al., 2012). Ho et al. (2007) described that the local activity of endothelial COX and FAAH was acting to blunt anandamide-induced vasodilator responses in small mesenteric arteries from rats. In cerebral arteries vasodilatation by anandamide and THC was abolished by indomethacin, implicating
the production of prostanoids in endocannabinoid responses (Ellis et al., 1995). Herradon et al. (2007) described an indomethacin-sensitive anandamide-induced vasorelaxation of rat aortae that was also sensitive to inhibition of FAAH. The response was driven by a COX-2 derived prostanoid active at endothelial prostaglandin EP₄ receptors. Other studies have also implicated the COX-pathway in responses to endocannabinoid (Pratt et al., 1998; Fleming et al., 1999; Grainger and Boachie-Ansah, 2001). In the present study indomethacin did not affect relaxation of the rat aortae to oleamide or the anandamide-induced vasorelaxation of porcine mesenteric arteries. This is in contrast to studies reported by O’Sullivan et al. (2005) in which indomethacin potentiated vasorelaxation to anandamide, THC and NADA in isolated aortae. Chapter 3 discusses the potentiation by indomethacin of oleamide-induced responses in WKY aortae.

Anandamide-induced vasorelaxation of porcine mesenteric arteries was abolished in the presence of the NO synthase inhibitor L-NAME. This demonstrates that anandamide can induce the release of NO, which acts to relax vascular smooth. Other studies have reported anandamide-induced release of NO (Deutsch et al., 1997; Poblete et al., 2005). Anandamide was shown to dilate perfused juxtaglomerular afferent arterioles, which was sensitive to the presence of L-NAME and rimonabant (Deutsch et al., 1997). The authors also demonstrated the CB₁-mediated release of NO in response to anandamide in perfused renal arterial segments. Interestingly, the vasorelaxant response to anandamide in porcine mesenteric arteries was not dependent on an intact endothelium. This would suggest that the NO released by anandamide is derived from a non-endothelial source. A hypothesis could be that anandamide is causing the release of neuronal NO. For example, in endothelium-denuded mesenteric arteries from the guinea pig it was demonstrated that electrical field stimulation induces vasodilatation
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(Gyoda et al., 1995). This response was attenuated with nitro-L-arginine and so was mediated by non-endothelial NO. Neuronal NO release induced by electrical field stimulation in SHR mesenteric arteries was increased by endogenous prostacyclin, demonstrating a regulatory role for neuronal NO in the vasomotor response (Ferrer et al., 2004). In the human sigmoid colon circular muscle capsaicin-induced relaxation was mediated by NO, as described by its sensitivity to the presence the NO synthase inhibitor L-NOARG (Bartho et al., 2002).

In summary, this chapter has demonstrated the vasorelaxant properties of oleamide in rat aortae. However, the vasorelaxant nature of oleamide was tissue dependent and oleamide did not affect isolated porcine coronary and mesenteric arteries. This chapter also reports differences between the mechanisms of action between oleamide and anandamide as the two substances did not elicit similar responses in any of the vessels tested. Oleamide-induced responses were regulated by an unidentified capsaicin-sensitive TRPV1-independent mechanism. Thus, results may suggest an additional site of action for oleamide.
Chapter 3

Enhanced vasorelaxant effects of oleamide and anandamide in hypertension
Chapter 3 Enhanced vasorelaxant effects of oleamide and anandamide in hypertension

3.1 Introduction

Endogenous cannabinoids and phytocannabinoids have recently received growing interest, partly because of their potential therapeutic use as antihypertensives (reviewed; Pacher et al., 2005; Sarzani, 2008; Cunha et al., 2011). In males, long-term smoking of marijuana has been linked to hypotensive effects that are diminished by rimonabant, a cannabinoid CB₁ receptor antagonist (Gorelick et al., 2006). The hypotensive effects of THC have been replicated in rat models, in which depressor effects are increased in spontaneously hypertensive rats compared to normotensive controls (Kosersky, 1978). Key evidence suggesting that the endocannabinoid system is a potentially important target for the treatment of hypertension comes from clinical trials of rimonabant. The clinical trials were designed to study the impact of rimonabant on weight loss in obese patients. Patients treated with rimonabant experienced significant weight loss (Despres et al., 2005). In addition, CB₁ receptor antagonism caused a decrease in blood pressure in obese patients with high blood pressure (Van Gaal et al., 2005; Pi-Sunyer et al., 2006; Rucker et al., 2007; Ruilopea et al., 2008), by a magnitude such that mortality associated with hypertension would be significantly decreased (Lewington et al., 2002). The decrease in blood pressure was presumably secondary to the weight loss caused by appetite suppression by antagonism of centrally located CB₁ receptors. However, treatment with rimonabant led to a number of adverse effects, including depression and increased anxiety (Leite et al., 2009). Despite these adverse effects, the case-study of rimonabant clearly highlights that inhibition of the central endocannabinoid system may result, albeit indirectly, in reductions in blood pressure.
It has been postulated that endocannabinoids may contribute towards the hypotensive state of certain pathophysiological diseases, including; haemorrhagic shock (Wagner et al., 1997), endotoxic shock (Varga et al., 1998; Wang et al., 2001; Liu et al., 2003; Batkai et al., 2004), cardiogenic shock (Wagner et al., 2001; 2003) and liver cirrhosis (Batkai et al., 2001; Ros et al., 2002; Domenicali et al., 2005). Recent research has implicated an upregulated endocannabinoid system, involving increased CB₁ expression and endocannabinoid production, in hepatic cirrhosis (Caraceni et al., 2010). Batkai et al. (2001) demonstrated that, in a rat model of cirrhosis, which is also hypotensive, the injection of rimonabant increased blood pressure. There was also an upregulation of CB₁ receptor expression in endothelial cells isolated from the vasculature (Batkai et al., 2001). Circulating monocytes from patients with chronic liver cirrhosis caused a decrease in blood pressure when injected into rats, characterised by increased levels of anandamide (Batkai et al., 2001). This further highlights the potential value of the endocannabinoid system in the regulation of blood pressure under pathophysiological conditions.

Both endocannabinoids and cannabinoids elicit enhanced depressor responses in animal models of hypertension in comparison to normotensive controls (Kosersky 1978; Lake et al., 1997; Batkai et al., 2004b; Li et al., 2003; Wang et al., 2005; Wheal et al., 2007). Batkai et al. (2004b) described enhanced depressor effects of anandamide in the anesthetised SHR compared to normotensive Wistar Kyoto rats (WKY). Increased depressor responses in the SHR were associated with the upregulation of aortic and cardiac CB₁ receptor expression (Batkai et al., 2004). The depressor effect of anandamide in the SHR does not appear dependent on anaesthesia (Wheal et al., 2007). The TRPV1 receptor has also been implicated in the depressor effects of anandamide (Li et al., 2003; Wang et al.,
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2005). Li et al. (2003) demonstrated that methanandamide, a stable analogue of anandamide, caused enhanced depressor effects in SHR rats, which was partially mediated by TRPV1 and CB1 receptors. The antihypertensive effect of anandamide was attenuated by capsazepine and in the mesenteric vasculature of the SHR there was an upregulation of CGRP receptor expression compared to the WKY (Li et al., 2003). Hence, anandamide-induced hypotension may be elevated in the SHR due to the activation of TRPV1 receptors on perivascular sensory nerves causing the release of CGRP. In conscious rats made hypertensive through a high salt diet, anandamide also caused enhanced hypotensive effects (Wang et al., 2005). The decreases in blood pressure induced by methanandamide were attenuated in the presence of either capsazepine or rimonabant (Wang et al., 2005). The blockade of both TRPV1 and CB1 receptors in combination completely abolished cardiovascular responses to methanandamide. The mesenteric vasculature from the high salt diet model of hypertension was associated with increased expression of TRPV1 receptors compared to the normotensive controls (Wang et al., 2005). Wang et al. (2007) demonstrated that methanandamide elicited a greater release of CGRP in arteries from hypertensive rats. In addition, plasma levels of anandamide were upregulated in rats made hypertensive by a high salt diet (Wang et al., 2007). It was postulated that anandamide levels are upregulated in order to limit blood pressure increases in high salt rat model through the activation of TRPV1 receptors and the subsequent release of CGRP (Wang et al., 2007).

The vasorelaxant properties of anandamide were also found to be enhanced in some isolated blood vessels from models of hypertension in comparison to vessels from normotensive controls. In a rat model of hypertension, where animals acquire hypertension
through chronic nitric oxide synthase inhibition with L-NAME, anandamide produced enhanced vasorelaxant effects in perfused mesenteric arterial beds (Mendizabel et al., 2001; Tep-Areenan et al., 2002; Wheal et al., 2007). Wheal and Randall (2009) demonstrated enhanced vascular responses to anandamide in two models of hypertension; the SHR and the L-NAME induced model of hypertension. In aortic rings from SHR rats the enhanced vasorelaxant response to anandamide was mediated by endothelium-dependent mechanisms which were independent of NO and CB₁ receptors (Wheal and Randall, 2009). Wheal and Randall (2009) similarly demonstrated greater relaxation of mesenteric arteries in the L-NAME model of hypertension and that the enhanced effects of anandamide were absent after pre-treatment of vessels with capsaicin, suggesting increased sensory nerve-mediated activity in hypertension. This is contradictory to previous research that showed capsazepine affected anandamide-induced responses equally in mesenteric beds from hypertensive and normotensive rats (Mendizabel et al., 2001). Interestingly, Wheal and Randall (2009) did not test the sensitivity of the anandamide response to capsazepine and Mendizabel et al. (2001) did not examine the effects of capsaicin pre-treatment.

In light of the research implicating the endocannabinoid system in hypertension, the hypotensive and antihypertensive nature of anandamide, and the vasorelaxant properties of oleamide, it is necessary to characterise the vascular effects of oleamide in hypertension, and compare with those of anandamide. The aim of this chapter was to investigate oleamide-induced vascular effects in isolated vessels from the SHR model of hypertension and compare them to the effects produced by anandamide.
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3.2 Methods and Materials

3.2.1 Animals

Male spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats (Charles River UK) (250-350g; aged 12-18 weeks) were used during this investigation. All rats used were housed at the Biomedical Services Unit, University of Nottingham with a 12h light/dark cycle and in temperature-controlled conditions.

3.2.2 Preparation of aortic rings and experimental protocol

The rats were killed and aortae removed and set-up following the protocols outlined in Chapter 2.

Vessels were pre-contracted with the $\alpha_1$-adrenoceptor agonist methoxamine (1-10µM) to achieve submaximal (~70%) contraction prior to the addition of oleamide (10nM-10µM) (Figure 3.1) or anandamide (1nM-10µM), which were added cumulatively at 5 minute intervals to construct concentration-response curves.

Figure 3.1

Figure 3.1 Representative trace of the response to oleamide in the SHR aorta. A= 10nM, B=30nM, C=100nM, D=300nM, E=1µM, F=3µM and G=10µM.
To investigate the role of sensory nerves, some vessels were subjected to pre-treatment with 10µM capsaicin for 1h (Zygmunt et al., 1999). Capsaicin pre-treatment was followed by a 20 minute wash-out prior to methoxamine contraction. Experiments were also performed in the presence of capsazepine (5µM), a TRPV1 receptor antagonist (White et al., 2001). To further analyse any differences in sensory nerve activity between SHR and WKY rats concentration-response curves to capsaicin (1nM-10µM) were constructed cumulatively. Experiments were also carried out in the presence of ruthenium red (10µM), a blocker of capsaicin-activated cation channels (Harris et al., 2002).

The contribution of the endothelium was assessed by denuding some vessels of endothelial cells. This was achieved by gentle rubbing of the intimal surface with metal forceps. Endothelial function was examined by addition of carbachol (10µM) after pre-contraction with methoxamine. Vessels responding with less than 20% relaxation of induced tone to carbachol were deemed to be lacking a functional endothelium. To inhibit nitric oxide synthase some vessels were incubated with L-NAME (300µM), while in other vessels indomethacin (10µM) was present to inhibit cyclo-oxygenase (O’Sullivan et al., 2004). Concentration-response curves to oleamide were also carried out in the presence of niflumic acid (10µM), a selective COX-2 inhibitor (Wheal et al., 2010). In some experiments the role of cannabinoid CB₁ receptors was examined by incubation with AM251 (1µM) a cannabinoid CB₁ receptor antagonist (O’Sullivan et al., 2005). Some experiments were carried out in the presence of URB597 (1µM), a fatty acid amide hydrolase (FAAH) inhibitor (Ho and Randall, 2007).
3.2.3 Drugs and reagents

Oleamide, anandamide and AM251 were obtained from Tocris Co. (UK). All other drugs used in this investigation were purchased from Sigma Chemicals Co. (UK). Anandamide, capsaicin and capsazepine were dissolved in ethanol at stock concentrations of 10 mM. DMSO was used to dissolve oleamide and AM251 at 10 mM and URB597 at 1 mM. All other drugs were dissolved in distilled water. L-NAME, indomethacin, AM251 and capsazepine were incubated for approximately 20 minutes before pre-contraction of vessels with methoxamine. Ruthenium red and niflumic acid were incubated for 30 minutes, while URB597 was incubated for 10 minutes before pre-contraction.

3.2.4 Statistical analysis

All responses are expressed as mean percentage vasorelaxation with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0 software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation \[ Y = \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_{50} - X) \cdot \text{Hillslope})}} + \text{Bottom} \], when \( X = \) logarithm of agonist concentration and \( Y = \) response from Bottom to Top in a sigmoidal shape. The curves were used to determine potency (\( EC_{50} \)) and maximal response (\( R_{\text{max}} \)) values. Potency (\( EC_{50} \)) is the negative log of agonist concentration that reduced methoxamine-induced contraction by 50%. The maximal response relates to the maximum percentage vasorelaxation of methoxamine-induced pre-contraction. Statistical significance was determined using two-tailed unpaired Student’s t-test between two data sets or one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. Statistical significance was determined using one-way
ANOVA unless stated otherwise in the figure legend. P-values <0.05 were considered significant.
3.3 Results

3.3.1 Vascular responses to oleamide and anandamide in isolated aortae from SHR and WKY rats

Oleamide caused concentration-dependent vasorelaxation of aortic segments from both SHR and WKY rats. Maximal relaxation was significantly (P<0.001) greater in SHRs ($R_{\text{max}}=40.3\pm3.5\%$, n=6) compared to that in aortae from WKY rats ($R_{\text{max}}=15.7\pm3.9\%$, n=6) (Figure 3.2). The potency of oleamide was comparable between aortae from SHR and WKY rats.

In aortic rings from SHR and WKY rats, anandamide also caused concentration-dependent vasorelaxation and maximal relaxation was significantly greater (P<0.001) in SHRs ($R_{\text{max}}=31.7\pm3.4\%$, n=10 SHR) compared to WKY rats ($R_{\text{max}}=11.8\pm0.5\%$, n=10 WKY rats), while the potency of anandamide was similar between strains ($pEC_{50}=5.85\pm0.45$, n=10 WKY rats; $pEC_{50}=6.36\pm0.28$, n=10 SHR) (Figure 3.3).
Figure 3.2 Vasorelaxant responses to oleamide in aortic rings pre-contracted with methoxamine from SHR and WKY rats. Mean data with bars indicating S.E.M displayed.
Figure 3.3

Figure 3.3 Vasorelaxant responses to anandamide in aortic rings pre-contracted with methoxamine from SHR and WKY rats. Mean data with bars indicating S.E.M displayed.
3.3.2 Effects of AM251, a cannabinoid CB₁ receptor antagonist, on the vascular responses to oleamide in aortae from SHR and WKY rats

The vasorelaxant responses caused by oleamide ($R_{\text{max}}=27.6\pm3.5\%$, $pEC_{50}=6.05\pm0.25$, n=8) were unaffected by the CB₁ receptor antagonist AM251 (1µM) in SHR aortae ($R_{\text{max}}=37.3\pm3.5\%$, $pEC_{50}=6.32\pm0.22$, n=6). Similarly, responses to oleamide ($R_{\text{max}}=13.1\pm3.2\%$, $pEC_{50}=5.80\pm0.40$, n=6) were unaffected in aortae from WKY rats in the presence of AM251 (1µM) ($R_{\text{max}}=15.8\pm1.7\%$, $pEC_{50}=5.90\pm0.21$, n=4) (Figure 3.4).

Figure 3.4 Vasorelaxant responses to oleamide in the presence of AM251 (1µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.3 Effects of AM251, a cannabinoid CB1 receptor antagonist, on the vascular responses to anandamide in aortae from SHR and WKY rats

Anandamide-induced vasorelaxation of aortae from both WKY ($R_{\text{max}}=12.3\pm1.6\%$, $p\text{EC}_50=6.71\pm0.31$, $n=6$) and SHR rats ($R_{\text{max}}=36.0\pm2.3\%$, $p\text{EC}_50=6.76\pm0.19$, $n=6$) was unaffected by the CB1 receptor antagonist AM251 (1µM) ($R_{\text{max}}=7.7\pm1.1\%$, $p\text{EC}_50=7.18\pm0.44$, $n=6$ WKY; $R_{\text{max}}=43.5\pm2.7\%$, $p\text{EC}_50=6.87\pm0.17$, $n=6$ SHR) (Figure 3.5).

Figure 3.5

Figure 3.5 Vasorelaxant responses to anandamide in the presence of AM251 (1µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.4 Effects of endothelial denudation on the vasorelaxant responses to oleamide in SHR and WKY aortae

The vasorelaxant effects of oleamide in both WKY ($R_{\text{max}}=13.1\pm3.2\%$, $\text{pEC}_{50}=5.80\pm0.40$, $n=6$) and SHR ($R_{\text{max}}=27.6\pm3.5\%$, $\text{pEC}_{50}=6.05\pm0.25$, $n=8$) arteries were unaffected by endothelial denudation ($R_{\text{max}}=10.8\pm2.2\%$, $\text{pEC}_{50}=6.20\pm0.44$, $n=6$ WKY; $R_{\text{max}}=23.2\pm5.0\%$, $\text{pEC}_{50}=5.72\pm0.32$, $n=9$ SHR) (Figure 3.6).

Figure 3.6

Figure 3.6 Vasorelaxation of endothelium denuded aortae from WKY and SHR rats by oleamide. Mean data with bars indicating S.E.M displayed.
3.3.5 Effects of L-NAME on the vasorelaxant responses to oleamide in SHR and WKY aortae

The vasorelaxation caused by oleamide in aortae from WKY ($R_{\text{max}}=13.9\pm4.5\%, \text{ pEC}_{50}=6.05\pm0.71, \ n=5$) and SHR ($R_{\text{max}}=40.3\pm3.5\%, \text{ pEC}_{50}=6.49\pm0.22, \ n=6$) rats was unaffected by L-NAME (300µM) ($R_{\text{max}}=14.6\pm1.7\%, \text{ pEC}_{50}=5.96\pm0.18, \ n=6$ WKY; $R_{\text{max}}=40.8\pm3.7\%, \text{ pEC}_{50}=6.14\pm0.19, \ n=6$ SHR) (Figure 3.7).

Figure 3.7

Figure 3.7: Vasorelaxant responses to oleamide in aortae from WKY and SHR rats in the presence of L-NAME (300µM). Mean data with bars indicating S.E.M displayed.
3.3.6 Effects of endothelial denudation on the vasorelaxant responses to anandamide in SHR and WKY aortae

Anandamide did not cause any vascular responses in isolated aortae from WKY rats. Anandamide-induced vasorelaxation of aortic segments from SHR rats ($R_{\text{max}}=26.4\pm2.8\%$, $pEC_{50}=6.97\pm0.29$, $n=7$) was unaffected by removal of the endothelium ($R_{\text{max}}=19.3\pm1.8\%$, $pEC_{50}=6.24\pm0.20$, $n=7$) (Figure 3.8).

Figure 3.8

![Graph showing vasorelaxation of endothelial denuded aortae from WKY and SHR rats by anandamide. Mean data with bars indicating S.E.M displayed.](image-url)
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3.3.7 Effects of L-NAME on the vasorelaxant responses to anandamide in SHR and WKY aortae

Anandamide failed to induce vasorelaxation in WKY aortae. However, vasorelaxation of SHR aortae by anandamide ($R_{\text{max}}=31.7\pm3.7\%$, $\text{pEC}_{50}=6.36\pm0.28$, $n=10$) was unaffected by L-NAME ($R_{\text{max}}=32.2\pm3.0\%$, $\text{pEC}_{50}=6.28\pm0.21$, $n=7$) (Figure 3.9).

Figure 3.9

![Vasorelaxant responses to anandamide in aortae from WKY and SHR rats in the presence of L-NAME (300µM). Mean data with bars indicating S.E.M displayed.](image)

Figure 3.9 Vasorelaxant responses to anandamide in aortae from WKY and SHR rats in the presence of L-NAME (300µM). Mean data with bars indicating S.E.M displayed.
3.3.8 Effects of cyclooxygenase inhibition on the vasorelaxant responses to oleamide in isolated aortae from SHR and WKY rats

Vasorelaxation to oleamide in SHR arteries ($R_{max}=40.3\pm3.5\%$, $pEC_{50}=6.49\pm0.22$, $n=6$) was unaffected by 10µM indomethacin ($R_{max}=28.7\pm3.8\%$, $pEC_{50}=5.90\pm0.28$, $n=6$) (Figure 3.10). In contrast in aortae from WKY control rats vasorelaxation to oleamide was significantly ($P<0.001$) enhanced in the presence of 10µM indomethacin, such that relaxation at 10µM oleamide was $37.3\pm3.6\%$ ($n=7$) compared to a relaxation of $15.5\pm4.5\%$ ($n=6$) at this concentration in the absence of indomethacin (Figure 3.10). Vasorelaxation to oleamide in both WKY ($R_{max}=13.1\pm6.2\%$, $pEC_{50}=5.65\pm0.57$, $n=6$) and SHR aortae ($R_{max}=43.3\pm4.4\%$, $pEC_{50}=6.04\pm0.18$, $n=6$) was unaffected by the COX-2 inhibitor niflumic acid (10µM) ($R_{max}=22.9\pm11.3\%$, $pEC_{50}=5.40\pm0.58$, $n=6$ WKY; $R_{max}=41.2\pm6.6\%$, $pEC_{50}=5.86\pm0.27$, $n=5$ SHR) (Figure 3.11).
Figure 3.10

Figure 3.10 Vasorelaxant effects of oleamide in WKY and SHR aortae in the presence of indomethacin (10µM). Mean data with bars indicating S.E.M displayed.
Figure 3.11 Vasorelaxant responses to oleamide in the presence of niflumic acid (10µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.9 Effects of cyclooxygenase inhibition on the vasorelaxant responses to anandamide in isolated aortae from SHR and WKY rats

In aortae from SHR rats the vasorelaxant responses caused by anandamide ($R_{\text{max}}=36.0\pm2.3\%$, $pEC_{50}=6.76\pm0.19$, $n=6$ SHR) were significantly reduced in the presence of indomethacin such that $R_{\text{max}}=19.9\pm2.8\%$ ($n=6$) (Figure 3.12), although potency was unaffected ($pEC_{50}=7.49\pm0.51$, $n=6$). In aortae from WKY rats the relaxant responses ($R_{\text{max}}=12.1\pm1.5\%$, $pEC50=6.86\pm0.28$, $n=6$) were completely abolished (Figure 3.12).

Figure 3.12 Vasorelaxant responses to anandamide in the presence of indomethacin (10µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.10 Effects of endothelial denudation on vascular responses to oleamide in the presence of indomethacin in WKY aortae

The enhanced vasorelaxant responses in WKY aortae to oleamide in the presence of 10µM indomethacin were abolished by the removal the endothelium (Figure 3.13).

Figure 3.13

Figure 3.13 Vasorelaxant effects of oleamide in endothelium denuded aortae from WKY rats in the presence of indomethacin (10µM). Mean data with bars indicating S.E.M displayed.
3.3.11 Effects of the FAAH inhibitor, URB597, on vasorelaxant responses to oleamide in aortae from SHR and WKY rats

Vasorelaxation to oleamide in both WKY ($R_{\text{max}}=6.3\pm3.7\%$, $pEC_{50}=5.64\pm0.56\ n=7$) and SHR ($R_{\text{max}}=43.2\pm7.1\%$; $pEC_{50}=6.13\pm0.32\ n=6$) aortae was unaffected by URB597 (1µM) ($R_{\text{max}}=6.3\pm5.3\%$, $pEC_{50}=5.60\pm0.66\ n=7$ WKY; $R_{\text{max}}=37.3\pm5.8\%$, $pEC_{50}=5.87\pm0.25\ n=6$ SHR) (Figure 3.14).

Figure 3.14

Figure 3.14 Vasorelaxant responses to oleamide in the presence of URB597 (1µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.12 Effects of the FAAH inhibitor, URB597, on vasorelaxant responses to anandamide in aortae from SHR and WKY rats

Vasorelaxation to anandamide in both WKY ($R_{\text{max}}=16.2\pm3.3\%$, $pEC_{50}=6.50\pm0.44$, $n=8$) and SHR ($R_{\text{max}}=25.3\pm3.3\%$; $pEC_{50}=6.84\pm0.45$, $n=6$) aortae was unaffected by URB597 (1µM) ($R_{\text{max}}=13.6\pm3.0\%$, $pEC_{50}=6.37\pm0.38$, $n=8$ WKY; $R_{\text{max}}=31.0\pm2.9\%$, $pEC_{50}=7.01\pm0.34$, $n=6$ SHR) (Figure 3.15).

Figure 3.15

[Graph showing vasorelaxant responses to anandamide in the presence of URB597 (1µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.]
Chapter 3 Enhanced vasorelaxant effects of oleamide and anandamide in hypertension

3.3.13 Effects of capsaicin pre-treatment on the vasorelaxant responses to oleamide in aortae from SHR and WKY rats.

Vasorelaxation to oleamide was significantly (P<0.001) reduced in the SHR arteries after pre-treatment with capsaicin for 1h ($R_{\text{max}}=9.8\pm1.5\%$, n=5, SHR), compared to the control value given above. The potency of the oleamide as a vasorelaxant was unaffected. Pre-treatment with capsaicin caused maximal responses to oleamide in the SHR to become comparable to the responses in WKY rats such that relaxation at 10µM oleamide was 9.5±2.0% (n=5) compared to a relaxation of 7.6±2.8% (n=6) (Figure 3.16).

Figure 3.16

Figure 3.16 Vasorelaxant responses to oleamide in aortic rings from WKY and SHR rats after a 1h pre-treatment of vessels with capsaicin (10µM). Control SHR and WKY data from Figure 3.2 included as a comparison and displayed as a dashed line. Mean data with bars indicating S.E.M displayed.
3.3.14 Effects of capsaicin pre-treatment on vasorelaxant response to anandamide in aortae from SHR and WKY rats

After pre-treatment with capsaicin the maximal response to anandamide was significantly (P<0.05) reduced ($R_{\text{max}}=19.5\pm1.6\%$, $n=7$, SHR), and its potency was also significantly (P<0.01) increased ($pE_{50}=8.44\pm0.55$, $n=7$, SHR) (Figure 3.17). Importantly, capsaicin pre-treatment caused the maximal responses to anandamide in SHR to become comparable with responses in the WKY ($R_{\text{max}}=22.1\pm3.6\%$, $n=7$) (Figure 3.17).

Figure 3.17

![Figure 3.17](image_url)

Figure 3.17 Vasorelaxant responses to anandamide in aortic rings from WKY and SHR rats after a 1h pre-treatment of vessels with capsaicin (10µM). Control SHR data from Figure 3.3 included as a comparison and displayed as a dashed line. Mean data with bars indicating S.E.M displayed.
3.3.15 Vasorelaxant effects of capsaicin in aortae from SHR and WKY rats

The vasorelaxant responses to capsaicin were comparable in aortic rings from both SHR and WKY rats such that at 10µM capsaicin vasorelaxation in SHR aortae was 30.4±5.3% (n=9) compared to 38.0±9.2% (n=8) (Figure 3.18).

Figure 3.18

Figure 3.18 Vasorelaxation caused by capsaicin in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
3.3.16 Effects of the TRPV1 antagonist capsazepine and ruthenium red on vasorelaxant responses to oleamide in aortae from SHR and WKY rats

The responses to oleamide in WKY arteries ($R_{max}=6.3\pm3.7\%$, $pEC_{50}=5.64\pm0.56$, $n=7$) and SHR arteries ($R_{max}=43.2\pm7.1\%$, $pEC_{50}=6.13\pm0.33$, $n=6$) were unaffected by capsazepine ($R_{max}=20.3\pm7.5\%$, $pEC_{50}=5.34\pm0.47$, $n=7$ WKY; $R_{max}=37.7\pm5.5\%$, $pEC_{50}=6.27\pm0.35$, $n=6$ SHR) (Figure 3.19). Vasorelaxation to oleamide in aortic rings from both WKY ($R_{max}=6.3\pm3.7$, $pEC_{50}=5.64\pm0.56$, $n=7$) and SHRs ($R_{max}=43.2\pm7.1\%$, $pEC_{50}=6.13\pm0.33$, $n=6$) was unaffected by 10µM ruthenium red ($R_{max}=10.3\pm2.6\%$, $5.68\pm0.30$, $n=7$ WKY; $R_{max}=42.6\pm6.35\%$, $pEC_{50}=5.71\pm0.23$, $n=6$ SHR) (Figure 3.20). In the presence of capsazepine some vessels from WKY rats were less sensitive to methoxamine. A range of 1µM-100µM of methoxamine was used to pre-contract these vessels.
Figure 3.19

Figure 3.19 Vasorelaxant effects of oleamide in WKY and SHR aortae in the presence of capsazepine (5µM). Mean data with bars indicating S.E.M displayed.
Figure 3.20 Vasorelaxant effects of oleamide in WKY and SHR aortae in the presence of ruthenium red (10µM). Mean data with bars indicating S.E.M displayed.
3.3.17 Effects of the TRPV1 antagonist capsazepine and ruthenium red on vasorelaxant responses to anandamide in aortae from SHR and WKY rats

The vasorelaxant effects of anandamide in aortae from WKY rats ($R_{\text{max}}=16.2\pm3.3\%$, $pEC_{50}=6.50\pm0.44$, $n=8$) were unaffected by 5µM capsazepine ($R_{\text{max}}=19.7\pm7.6\%$, $pEC_{50}=7.56\pm0.34$, $n=7$) (Figure 3.21). Similarly, vasorelaxation of SHR arteries ($R_{\text{max}}=25.3\pm3.3\%$, $pEC_{50}=6.84\pm0.45$, $n=6$) was unaffected by 5µM capsazepine ($R_{\text{max}}=30.9\pm4.1\%$, $pEC_{50}=6.77\pm0.39$, $n=5$) (Figure 3.21). The vasorelaxant effects of anandamide were unaffected in the presence of 10µM ruthenium red in both WKY and SHR arteries ($R_{\text{max}}=10.9\pm1.9\%$, $pEC_{50}=6.13\pm0.29$, $n=7$ WKY; $R_{\text{max}}=29.6\pm3.9\%$, $pEC_{50}=7.15\pm0.52$, $n=5$ SHR) (Figure 3.22).
Figure 3.21

Figure 3.21 Vasorelaxant effects of anandamide in WKY and SHR aortae in the presence of capsazepine (5µM). Mean data with bars indicating S.E.M.
Figure 3.22 Vasorelaxant effects of anandamide in WKY and SHR aortae in the presence of ruthenium red (10µM). Mean data with bars indicating S.E.M displayed.
3.4 Discussion

The principal finding of this chapter was that the vasorelaxant responses to the endocannabinoid-like substance, oleamide are substantially enhanced in the aorta isolated from the SHR model of hypertension and that this may be related to alterations in prostanoid metabolism. This study was designed as a comparison between the effects of the prototypical endocannabinoid, anandamide, with those of oleamide in hypertension. A clear finding from this study was that the enhanced effects of both oleamide and anandamide in SHR aortae were capsaicin-sensitive. Indeed, previous studies have associated increased sensory nerve activity with models of hypertension (Li et al., 2003; Li and Wang, 2003; Wang et al., 2005). The magnitude of the vasorelaxant responses to oleamide in aortae from the hypertensive strain are comparable to those caused by anandamide, approximately 30-40% relaxation of induced tone.

Anandamide is known to cause vasorelaxation via stimulating vanilloid receptors on sensory nerves (Zygmunt et al., 1999). Furthermore, sensory nerves via TRPV1 activation are also involved in vasorelaxation caused by oleamide in small mesenteric arteries from normotensive Wistar rats (Sudhahar et al., 2009). In Chapter 2, oleamide-induced vasorelaxation of aortae was found to be sensitive to capsaicin pre-treatment. In a previous study, in the L-NAME-induced model of hypertension, the enhanced vasorelaxant effects of anandamide in mesenteric beds were normalised by capsaicin pre-treatment and so were ascribed to an upregulation of sensory-nerve mediated activity (Wheal and Randall, 2009). This could possibly be explained by an increase in TRPV1 expression in the mesenteric vasculature in hypertensive rats (Wang et al., 2005). Wheal and Randall (2009) described the effects of two different models of hypertension on the vasorelaxant effects of anandamide.
In support of the current findings anandamide-induced vasorelaxation was enhanced in aortae from SHR rats, this endothelium-dependent effect of anandamide was insensitive to capsaicin pre-treatment of aortae (Wheal and Randall, 2009). However, in mesenteric arterial beds from SHR rats, anandamide was less potent as a vasorelaxant and was associated with impaired NO-dependent relaxations (Wheal and Randall, 2009). This contradicts earlier reports by Li et al. (2003) that demonstrated enhanced depressor responses to methanandamide in SHRs was partly due to activation of sensory nerves. The enhanced depressor effect was associated with increased CGRP expression in the mesenteric vasculature that presumably increased vascular sensitivity to methanandamide in the SHR (Li et al., 2003).

In the present study the enhanced responses to both oleamide and anandamide were sensitive to capsaicin pre-treatment and the resulting responses were comparable to those in the normotensive controls. This is clearly consistent with the proposal that the enhanced responses in the SHR arteries are due to increased sensory nerve activity. However, when additional pharmacological approaches were used, including the TRPV1 antagonist capsazepine and the non-selective cation channel blocker, ruthenium red, the enhanced responses were unaffected. This suggests that although the mechanism underlying the enhanced responses to endocannabinoids is capsaicin-sensitive, it does not appear to involve TRPV1 channel activation or sensory nerves. Indeed, the vasorelaxant responses to capsaicin alone were comparable between the hypertensive and normotensive strains. This may suggest that there is comparable sensory nerve activity between the strains or alternatively that capsaicin is acting via mechanisms distinct from the sensory nerve-mediated pathway. Indeed, the previous chapter describes studies conducted with arteries from Wistar rats that
demonstrate that capsaicin inhibits responses to oleamide in a sensory nerve independent manner. Chapter 6 demonstrates that capsaicin itself causes vasorelaxation possibly via the direct or indirect inhibition of calcium influx through L-type calcium channels.

Increased CB1 expression was the original hypothesis for the augmented responses to oleamide in SHR aortic segments. This was based on literature showing that the aortic endothelium from SHRs is associated with greater CB1 expression (Batkai et al., 2004), coupled with the ability of oleamide to act as an endogenous CB1 receptor agonist (Leggett et al., 2004). The involvement of CB1 receptors has been reported in oleamide-induced vasorelaxation of small mesenteric arteries (Sudhahar et al., 2009). Anandamide is also a partial agonist at CB1 receptors (Pertwee, 2005) and in anesthetised mice anandamide induces hypotensive effects, which are absent in CB1 receptor knock-out models (Ledent et al., 1999). In anesthetised rats anandamide also reduces blood pressure in a CB1 receptor dependent manner (Malinowska et al., 2001). Batkai et al. (2004) reported enhanced depressor responses to anandamide in hypertension, effects that were blocked by CB1 antagonism. In addition, CB1 antagonists were found to cause pressor responses in SHR rats (Batkai et al., 2004). However, in the present study, the enhanced vasorelaxation to both oleamide and anandamide was unaffected by the presence of the CB1 receptor antagonist, AM251, and are therefore independent of CB1 receptor activation. While, Batkai et al. (2004) described increased expression of CB1 in the aortic endothelium, the augmented vasorelaxation of SHR aortae by anandamide reported elsewhere was similarly insensitive to the presence of AM251 (Wheal and Randall, 2009). Many of the vascular effects of anandamide in previous studies occur independently of CB1 receptors. In isolated aortae from Wistar rats, vasorelaxant responses to anandamide and NADA were insensitive
to CB$_1$ receptor antagonism (O’Sullivan et al., 2005; Herradon et al., 2007).

The presence of indomethacin increased the vasorelaxant effects of oleamide in normotensive aortae, such that they were comparable to those from SHR. This clearly highlights the importance of the cyclooxygenase pathway in regulating the responses to oleamide. In addition to these findings, other studies have shown that COX-inhibition potentiates the vasorelaxant responses to endocannabinoids (Ho and Randall, 2007). Ho and Randall (2007) described relaxant responses to the endocannabinoids, anandamide and 2-AG, in small mesenteric arteries from wistar rats. The anandamide-induced responses were augmented by the presence of the COX-2 inhibitor nimesulide and also URB597. The relaxant responses to 2-AG were similarly increased by indomethacin and flurbiprofen, which is more potent at COX-1, and were augmented by an inhibitor of both FAAH and MGL (Ho and Randall, 2007). Ho and Randall (2007) suggest a regulatory action of the FAAH, MGL and COX pathways limits endocannabinoid-induced vasorelaxation. With COX-1 implicated in 2-AG-induced responses and COX-1 in anandamide induced responses. It is important to note that anandamide can be hydrolysed by FAAH into arachidonic acid metabolites that can be further metabolised by the COX-pathway (Fowler et al., 2007), or alternatively can be directly metabolised by cyclo-oxygenase enzymes into vasoactive prostanoids. Interestingly, vasorelaxant responses to anandamide and NADA in the Wistar rat isolated aorta were also enhanced by the inhibition of the COX pathway (O’Sullivan et al., 2005). This suggests that the regulation of endocannabinoid-induced responses by the COX pathway may be widespread in the normotensive vasculature. The increased responses to anandamide and 2-AG in the presence of COX-inhibition in mesenteric arteries was dependent on an intact
endothelium (Ho and Randall, 2007). Similarly, in the present study the enhanced response to oleamide in WKY aortae in the presence of indomethacin was absent in endothelium denuded vessels. Vasorelaxation of perfused mesenteric arterial beds by N-oleoylethanolamine, an endocannabinoid-like substance, is also augmented by the presence of indomethacin (Wheal et al., 2010).

It is unlikely that oleamide is a direct substrate for an upregulated COX-pathway. Therefore, metabolism of oleamide could act to limit vasorelaxation in aortic rings from normotensive rats and so accounting for the enhanced effects in SHR aortae. Inhibition of the COX-pathway with indomethacin would then remove the modulation of vasorelaxation to oleamide in the WKY and responses are normalised between the two strains. Alternatively, it is possible that vasoconstrictor prostanoids are being released in WKY aortae in response to oleamide and removal of these prostanoids would enhance vasorelaxation. A possible example of this mechanism in action is in the vasorelaxation of rat mesenteric arterial beds by N-oleoylethanolamine, which was sensitive to capsaicin pre-treatment and potentiated by indomethacin. The effect of indomethacin on N-oleoylethanolamine-induced responses was replicated by flurbiprofen and vapiprost, a thromboxane A₂ receptor antagonist (Wheal et al., 2010). This suggests that the release of vasoconstrictive prostanoids via COX-1 acting at thromboxane A₂ limits N-oleoylethanolamine-induced responses.

The production of any vasoconstrictor prostanoids is unlikely to be coupled to FAAH-dependent metabolism of oleamide as the responses were unaffected by the presence of URB597. Furthermore, oleic acid, which is the metabolite of FAAH-degradation of oleamide, is an unlikely substrate for COX-enzymes. The upregulation of responses to oleamide is independent of COX-2
Chapter 3 Enhanced vasorelaxant effects of oleamide and anandamide in hypertension

as the presence of niflumic acid had no effect on the vasorelaxant response to oleamide in WKY aortic rings.

The current study shows enhanced vasorelaxant responses to anandamide in aortae from SHR rats, which is consistent with the responses reported in Wheal and Randall (2009). In both strains the anandamide-induced responses were sensitive to the presence of indomethacin, used to inhibit the cyclo-oxygenase pathway. This is consistent with the actions of anandamide reported in the isolated aorta from the normotensive wistar rat (Herradon et al., 2007). Herradon et al. (2007) described a vasorelaxant effect of anandamide that was sensitive to both FAAH and COX-inhibition. It was suggested that the production of the COX-2 derived prostaglandin E$_2$ was involved in the vasorelaxant response to anandamide. In the present study, any production of vasoactive prostanoids is independent of FAAH-mediated degradation of anandamide as vasorelaxation persists in the presence of URB597. It is possible that anandamide could be metabolised directly by COX-2 (Yu et al., 1997). It can be concluded that the cyclo-oxygenase pathway is an important mechanism underlying anandamide-induced responses in both strains. However, in the presence of indomethacin, vasorelaxation to anandamide continued to be greater in SHR aortae and so is not the result of an upregulated cyclo-oxygenase pathway. It is also possible that indomethacin may be able to augment responses to oleamide independently of the COX-pathway. One hypothesis to explain the augmented responses which occurs in the SHR that are uncovered in the WKY arteries following COX inhibition could be that oleamide mobilises endogenous arachidonic acid. The liberated arachidonic acid could then enter the COX pathway. It could be that COX-dependent metabolism of arachidonic acid to opposing vasoconstrictor prostanoids occurs in the WKY arteries to limit
vasorelaxation and that this modulation is absent or impaired in SHR arteries. In this respect it has been shown previously that vasorelaxation to \( N \)-oleoylethanolamine, which cannot be metabolised to arachidonic acid, is also enhanced by COX inhibition and antagonism of thromboxane receptors (Wheal et al., 2010). This is consistent with increased vasoconstrictor prostanoid activity opposing vasorelaxation.

In conclusion, this chapter reports greatly enhanced vasorelaxant effects of the endocannabinoid-like substance oleamide and anandamide in aortae from the SHR model of hypertension. The augmented responses to both oleamide and anandamide were both abolished by capsaicin pre-treatment but are independent of TRPV1 receptor activity. An important finding was that vasorelaxant responses to oleamide are normalised by the presence of indomethacin in aortae. Therefore, the COX-pathway is an important component in regulating oleamide-induced vasorelaxation in normotensive aortae and this is lost in hypertension. It is possible that this is an adaptive change to modulate the rise in blood pressure.
Chapter 4

Effects of hypertension on endothelium-dependent vasorelaxation of the aorta
Chapter 4 Effects of hypertension on endothelium-dependent vasorelaxation of the aorta

4.1 Introduction

Certain pathological conditions including hypertension (Taddei et al., 1996; Hedner et al., 1997; Khder et al., 1998; Park et al., 2001) and diabetes (reviewed in De Vriese et al., 2000; Balletshofer et al., 2000; Stehouwer et al., 2002; Park et al., 2008; Matsumoto et al., 2010) are associated with altered endothelial function. In the past, research has demonstrated that endothelium-dependent vasorelaxation is blunted in aortae isolated from SHR rats compared to normotensive controls (Luscher and Vanhoutte, 1986; Auch-Schwelk et al., 1990; Yang et al., 2002; Gluais et al., 2005). The endothelial dysfunction associated with hypertension is mediated by the production of endothelium-derived contracting factors (EDCF) as opposed to the absence or attenuation of a relaxing factor (Luscher and Vanhoutte, 1986). Luscher and Vanhoutte (1986) demonstrated that endothelium dependent relaxation of SHR aortae from rats ages 30-34 weeks was normalised by the presence of indomethacin, thus implicating the production of COX-derived vasoconstrictive prostanoids. EDCF mediated responses in SHR aortae correlates with an upregulated endothelial COX-1 expression (Ge et al., 1995; Tang and Vanhoutte, 2008). Gluais et al., (2005) demonstrated that aortae from 1-year old SHRs released increased amounts of prostacyclin, which can act as a vasoconstrictor under certain conditions. Endothelium-dependent contractions are also sensitive to the presence of TP-receptor antagonists (Auch-Schwelk et al., 1990; Kato et al., 1990; Yang et al., 2002) present on vascular smooth muscle (Yang et al., 2003). The association between hypertension and endothelial function has also been demonstrated in patients with essential hypertension (Hedner et al., 1997; Khder et al., 1998; Kimura et al., 1999) and in other models.
of hypertension, including in renal mass reduction rats (Kimura and Nishio, 1999).

Therefore, according to the literature, endothelial dysfunction in hypertension may be related to the increased release of COX-derived vasoconstrictive prostanoids, including prostacyclin, diffusing to the vascular smooth muscle to activate TP-receptors. However, Wheal and Randall (2009) reported enhanced endothelium-dependent vasorelaxation of SHR aortae to carbachol. Authors used aortae from 20-week old SHRs and other investigations also report intact or enhanced endothelial function in rats that are younger than those used in Luscher and Vanhoutte (1986) and represent an earlier stage of established hypertension. In rats 12-15 weeks of age, it was shown that mesenteric vasodilatation induced by carbachol was comparable between SHR and WKY rats. In similarly aged SHRs (11-13 weeks) EDHF-mediated responses in the caudal artery were intact (Sandow et al., 2003). Another clear demonstration of intact endothelial function in younger SHRs was reported by Radaelli et al. (1998). In SHRs aged 12 weeks pressor responses induced by the inhibition of nitric oxide synthase were enhanced compared to WKY rats, possibly suggesting augmented NO activity in the hypertensive strain. Interestingly, in 6 week old SHRs that were pre-hypertensive pressor responses were similar between the two strains (Radaelli et al., 1998). In 8-12 week SHRs an increase in the activity of vascular NOS was reported that correlated with greater amounts of plasma NO metabolites (Vaziri et al., 1998). In addition, endothelium-dependent renal vasodilatation induced by acetylcholine remained intact and responses to arachidonic acid were enhanced in SHRs aged 13-15 weeks (Pomposiello et al. 2001). In (mREN-2)-27 transgenic rat model of hypertension intact responses to carbachol in mesenteric arterial beds were also described (Randall and March,
However, the inhibition of NO production or EDHF attenuated responses to a greater degree in normotensive controls in comparison to hypertensive rats (Randall and March, 1998). This points to the augmentation of the compensatory relationship between NO and EDHF in the transgenic model of hypertension.

Similarly, to results presented in Chapter 3, Wheal and Randall (2009) also described augmented responses to anandamide in SHR aortae. In light of these differences and the similarity of anandamide-induced responses with Wheal and Randall (2009), it is necessary to characterise the endothelial function in SHR aortae. Moreover, in Chapter 3 the COX-pathway was an important factor in limiting oleamide-induced vasorelaxation in normotensive aortae. This presents the possibility that a difference exists between the endothelial function of WKY and SHR aortae.
4.2 Methods and Materials

4.2.1 Animals

Male SHR and WKY rats (Charles River UK) (250-350g; aged 12-18 weeks) were used during this investigation. All rats used were housed at the Biomedical Services Unit, University of Nottingham with a 12h light/dark cycle and in temperature-controlled conditions.

4.2.2 Preparation of aortic rings and experimental protocol

The rats were killed, aortae removed and set-up following the protocols outlined in Chapter 2. Endothelium-dependent relaxation was investigated by constructing concentration-response curves for carbachol (1nM-10µM) in SHR (Figure 4.1) and WKY aortae.

Figure 4.1

![Representative trace of the response to carbachol in the SHR isolated aorta. A=1nM, B=3nM, C=10nM, D=30nM, E=100nM, F=300nM, G=1µM, H=3µM and I=10µM.](image)

The vasorelaxant effects of carbachol were also examined in the presence of L-NAME (300µM), indomethacin (10µM), catalase (1000 units/ml) or after a 1h capsaicin (10µM) pre-treatment. Catalase
was used in order to establish the contribution of hydrogen peroxide to carbachol responses in SHR aortae. The contribution of gap junctions to carbachol responses was also investigated by constructing concentration-response curves for carbachol (1nM-10µM) in SHR aortae in the presence of carbenoxolone (100µM) alone and together with L-NAME (300µM). The effect of a depolarising concentration of extracellular potassium (60mM) (Hoi and Hiley, 2006), alone and in combination with L-NAME and a 1h capsaicin pre-treatment, on carbachol concentration-response curves was also examined in order to determine the importance of K⁺ channels in responses in SHR aortae. These experiments were performed in an attempt to characterise a NO-independent vasorelaxant response to carbachol in aortae from SHR rats.

4.2.3 Drugs and reagents

Carbenoxolone (3β-Hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate), carbachol ((2-Hydroxyethyl) trimethylammonium chloride carbamate), capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide), L-NAME (NG-Nitro-L-arginine methyl ester hydrochloride), methoxamine (α-(1-Aminoethyl)-2, 5-dimethoxybenzyl alcohol hydrochloride), indomethacin (1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid) and catalase were purchased from Sigma Chemical Co. (UK). Capsaicin and indomethacin were dissolved in ethanol at stock concentrations of 10mM. All other drugs were dissolved using distilled water. Catalase and carbenoxolone were present in organ baths 1h prior to the addition of methoxamine. L-NAME and indomethacin were added approximately 20 minutes before methoxamine.
4.2.4 Statistical analysis

All responses are expressed as mean percentage vasorelaxation with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0 software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC50} - X) \cdot \text{Hillslope}}}, \]

where \( X = \) logarithm of agonist concentration and \( Y = \) response from Bottom to Top in a sigmoidal shape. The curves were used to determine potency (pEC\(_{50}\)) and maximal response (R\(_{max}\)) values. Potency (pEC\(_{50}\)) is the negative log of agonist concentration that reduced methoxamine-induced contraction by 50%. The maximal response relates to the maximum percentage vasorelaxation of methoxamine-induced pre-contraction. Statistical significance was determined using two-tailed unpaired Student’s t-test between two data sets or one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. Statistical significance was determined using one-way ANOVA unless stated otherwise in the figure legend. P-values <0.05 were considered significant.
4.3 Results

4.3.1 Endothelium-dependent vasorelaxant responses to carbachol in aortae from SHR and WKY rats

Carbachol caused concentration-dependent vasorelaxation in aortic rings from both SHR and WKY rats. The maximal response produced in SHR arteries ($R_{\text{max}} = 66.6 \pm 4.2\%$, $n=10$) was significantly greater ($P<0.001$) compared to that in the WKY arteries ($R_{\text{max}} = 37.1 \pm 2.7\%$, $n=10$) (Figure 4.2). The potency of carbachol was similar in the SHR and WKY arteries ($p\text{EC}_{50} = 6.24 \pm 0.13$, $n=10$ WKY rats; $p\text{EC}_{50} = 6.54 \pm 0.14$, $n=10$ SHR) (Figure 4.2)

Figure 4.2

Figure 4.2 Vasorelaxant responses to carbachol in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
4.3.2 Effects of L-NAME on the vascular responses to endothelium-dependent vasorelaxant carbachol in aortae from SHR and WKY rats

The vasorelaxant effects of carbachol were abolished in the presence of 300µM L-NAME in aortae from WKY rats. The maximal response to carbachol in SHR aortae was significantly (P<0.001) reduced in the presence of 300µM L-NAME ($R_{\text{max}}=31.0\pm3.5\%$, n=6) (Figure 4.3).

Figure 4.3

Figure 4.3 Vasorelaxant responses to carbachol in the presence of L-NAME (300µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed. Dashed lines represent SHR and WKY control data taken from Figure 4.2.
4.3.3 Effects of indomethacin on the vascular responses to carbachol in aortae from SHR and WKY rats

The maximal relaxant response to carbachol ($R_{\text{max}}=87.1\pm3.3\%, n=7$ SHR; $R_{\text{max}}=67.3\pm4.2\%, n=4$ WKY) was significantly increased in the presence of 10$\mu$M indomethacin in both SHR preparations ($R_{\text{max}}=123\pm4.4\%, n=7$ SHR) ($P<0.001$) and WKY preparations ($R_{\text{max}}=93.1\pm2.8\%, n=5$ WKY) ($P<0.05$) (Figure 4.4). However, the potency of carbachol ($pEC_{50}=6.70\pm0.14$, $n=4$ WKY; $pEC_{50}=6.96\pm0.10$, $n=7$ SHR) remained unaffected by the presence of indomethacin in aortae from either WKY ($pEC_{50}=6.52\pm0.06$, $n=5$) and SHR rats ($pEC_{50}=6.79\pm0.09$, $n=7$) (Figure 4.4).
Figure 4.4 Vasorelaxant responses to carbachol in the presence of indomethacin (10µM) in aortae from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
4.3.4 Effects of capsaicin pre-treatment on the vascular responses to endothelium-dependent vasorelaxant carbachol in aortae from SHR and WKY rats

The vasorelaxant responses to carbachol were unaffected by a 1h capsaicin (10µM) pre-treatment (with 20min washout) in both SHR (R$_\text{max}$=66.0±5.8%, pEC$_{50}$=6.48±0.22, n=5) and WKY preparations (R$_\text{max}$=41.2±10.2%, pEC$_{50}$=5.84±0.40, n=6) (Figure 4.5).

Figure 4.5 Vasorelaxant responses to carbachol after a 1h pre-treatment with capsaicin (10µM) of aortae from WKY and SHR rats. Dashed lines display SHR and WKY control data from Figure 4.2. Mean data with bars indicating S.E.M displayed.
4.3.5 Effects of catalase, in the presence of L-NAME, on the vasorelaxant responses to carbachol in aortae isolated from SHR and WKY rats

The vasorelaxant responses to carbachol in aortae isolated from WKY rats ($R_{max}=72.0\pm3.05\%$, $pEC_{50}=6.44\pm0.09$, $n=4$) were abolished by the combined presence of catalase and L-NAME. The maximal vasorelaxant response to carbachol in aortae from SHR rats ($R_{max}=89.0\pm3.5\%$, $pEC_{50}=6.81\pm0.09$, $n=4$) was significantly ($P<0.001$) reduced in the presence of L-NAME and catalase ($R_{max}=32.5\pm5.2\%$, $pEC_{50}=7.33\pm0.52$, $n=4$), however the response were no different from those in the presence of L-NAME alone and the residual relaxation remained. The potency of carbachol was unaffected by the presence of catalase and L-NAME (Figure 4.6).
4.3.6 Effects of L-NAME, capsaicin pre-treatment and high extracellular potassium on the vasorelaxant responses to carbachol in SHR aortae

The maximal vasorelaxant response to carbachol in the presence of high extracellular K+ in aortae from SHR rats ($R_{\text{max}}=100.6\pm5.5\%$, pEC$_{50}=6.76\pm0.14$, n=3) was significantly (P<0.001) decreased by L-NAME ($R_{\text{max}}=43.9\pm4.8\%$, pEC$_{50}=6.86\pm0.28$, n=3), but the presence of L-NAME in addition to pre-treatment of vessels with capsaicin had no further effect on the vasorelaxant response to carbachol ($R_{\text{max}}=26.4\pm3.6\%$, pEC$_{50}=6.86\pm0.37$, n=3) (Figure 4.7).
Figure 4.7

- high K+ (n=3)
- L-NAME + high K+ (n=3)
- caps pre-treatment + L-NAME + high K+ (n=3)

Figure 4.7 Vasorelaxant responses to carbachol in the presence of high extracellular K+ (60mM) and L-NAME (300µM) in aortae from SHR rats. Contraction was initiated with High K+ (60mM). Mean data with bars indicating S.E.M displayed.

4.3.7 Effects of the gap junction inhibitor, carbenoxolone, on the vasorelaxant responses to carbachol in SHR aortae

The vasorelaxant responses to carbachol in SHR aortae ($R_{\text{max}}=78.4 \pm 6.9\%$, $\text{pEC}_{50}=6.73 \pm 0.19$, $n=4$) were unaffected by the gap junction inhibitor carbenoxolone ($R_{\text{max}}=90.3 \pm 6.7$, $\text{pEC}_{50}=6.76 \pm 0.17$, $n=4$). Similarly, the residual vasorelaxant responses produced by carbachol in the presence of L-NAME ($R_{\text{max}}=18.2 \% \pm 3.2$, $\text{pEC}_{50}=7.11 \pm 0.42$, $n=4$) were unaffected by
carbenoxolone ($R_{\text{max}}=26.2\pm4.4$, $\text{pEC}_{50}=7.41\pm0.56$, $n=4$) (Figure 4.8).

Figure 4.8

![Graph showing vasorelaxant responses to carbachol in the presence of carbenoxolone (100µM) and L-NAME (300µM) from SHR rats. Mean data with bars indicating S.E.M displayed.]

Figure 4.8 Vasorelaxant responses to carbachol in the presence of carbenoxolone (100µM) and L-NAME (300µM) from SHR rats. Mean data with bars indicating S.E.M displayed.
4.4 Discussion

This chapter demonstrates augmented endothelium-dependent responses in SHR aortae to carbachol. Responses to carbachol were sensitive to L-NAME in both strains. The enhanced endothelial responses in SHR aortae contradict early work that formed the dogma that the SHR is associated with impaired endothelial function (Luscher and Vanhoutte, 1986; Auch-Schwelk et al., 1990; Ito et al., 1991; Ge et al., 1995; Yang et al., 2002; Gluais et al., 2005).

Luscher and Vanhoutte (1986) described an augmented acetylcholine response in aortae from normotensive rats compared to those from SHRs. Indomethacin enhanced vasorelaxation in SHRs normalising the response in comparison to the control strain, through the inhibition of a prostanoid endothelium-derived contracting factor (EDCF). Therefore, the impaired endothelial function in hypertension was thought to be due to the release of an EDCF, which acts to blunt endothelium-dependent relaxations (Luscher and Vanhoutte, 1986). Prostacyclin (Rapoport et al., 1996; Gluais et al., 2005; 2007) and endoperoxides (Ito et al., 1991; Ge et al., 1995) have been implicated as EDCFs. It is noteworthy that COX-enzymes metabolise arachidonic acid into endoperoxides, which are subsequently synthesised into a range of prostanoids. EDCF-mediated responses are evoked by COX activation, possibly mediated via oxygen-free radicals (Yang et al., 2002) and diffuse from the endothelium (Yang et al., 2003) to activate smooth muscle TP receptors (Auch-Schwelk et al., 1990; Yang et al., 2004) causing contractile responses. However, in contrast to these reports the present study demonstrates enhanced endothelial function in SHR aortae. This finding is not without precedent as Wheal and Randall (2009) reported similarly enhanced carbachol-induced responses in SHR aortae, while other reports describe intact endothelium-dependent responses (Pomposiello et al., 2001; Sandow et al., 2003;
Ford and Rush, 2010). However, the discrepancies outlined regarding endothelial dysfunction in hypertension are likely to reflect age-related differences, Luscher and Vanhoutte (1986) used arteries from rats at 30-34 weeks of age, while the present study used SHR and WKY rats at 12-18 weeks and previous papers used SHRs at 12-15 (Randall et al., 1991) and 20 weeks (Wheal and Randall, 2009). Indeed, EDCF-mediated responses in SHR are positively correlated with age (Koga et al., 1988; 1989) and there are also age-dependent differences reported in the expression of aortic COX-enzymes, prostanoid receptors and prostanoid synthases in SHR rats (Tang and Vanhoutte, 2008) and this may cause the age-related differences in endothelial function of SHR aortae. Radaelli et al. (1998) demonstrated that NO activity in SHRs was intact at 12 weeks of age and may be enhanced as shown by augmented pressor responses to NO synthase inhibition in vivo. The present study describes enhanced endothelium-dependent responses during the early stages of established hypertension, while previous research reports an age-dependent endothelial dysfunction seen in the latter stages of hypertension due to upregulated production of EDCFs. The endothelium-dependent responses in the present study were enhanced in aortae from both strains by the presence of indomethacin. This may suggest that, in response to carbachol, there is no difference in the production of EDCFs, and so are enhanced by a similar magnitude by indomethacin. Alternatively, indomethacin may be enhancing the carbachol responses by different mechanisms. In WKY rats of the same age as used in the present study, indomethacin was shown to increase acetylcholine-induced responses by augmenting free-radical oxygen production, namely ONOO⁻ (De Angelis et al., 2004). The authors concluded that this effect of indomethacin occurred independently of the COX pathway as other COX inhibitors used failed to potentiate vasorelaxant responses.
There is evidence of enhanced endothelial function in models of hypertension, including an upregulation in the NO system (Radaelli et al., 1998; Chang et al., 2002). Radaelli et al. (1998) reported that pressor responses to the inhibition of NO synthesis with N\textsuperscript{\textgreek{g}}-monomethyl-L-arginine (L-NMMA) in SHR rats of 12 weeks of age in comparison to normotensive controls. Wheal and Randall (2009) demonstrated that enhanced vasorelaxation to anandamide in aortae from SHR rats was abolished by removal of the endothelium. In the present study, endothelium-dependent responses in the WKY aorta were abolished by L-NAME and therefore mediated by NO. However, in the SHR aorta there was a significant L-NAME insensitive component with a residual relaxation of approximately 30%. This residual component could be responsible for the augmented endothelial responses in SHR aortae. In an attempt to elucidate the mechanisms underlying this component the contributions of putative EDHF-type mechanisms such as gap junctions and hydrogen peroxide were assessed as was sensory nerve activity.

The residual relaxation to carbachol was not dependent on NO or COX-mediated mechanisms and the hypothesis was that the residual relaxation could be due to an EDHF component in SHR aortae. EDHF-activity is usually associated with small resistance vessels (Shimokawa et al., 1996; Tomioka et al., 1999) and was absent in the WKY aorta. Hydrogen peroxide, K\textsuperscript{+} ions and electrical communication between cells through myo-endothelial gap-junctions have all been implicated in EDHF-mediated responses. The involvement of K\textsuperscript{+} ion release (Edwards et al., 1996; Coleman et al., 2001; Nelli et al., 2003; Torondel et al., 2004; McNeish et al., 2005) and activation of hyperpolarising K\textsuperscript{+} channels (Corriu et al., 1996; Zygmunt et al., 1997; Walker et al., 2001) is well documented in EDHF-mediated responses. In this study the presence of High K\textsuperscript{+}
and L-NAME had no effect on the residual relaxation of SHR aortae by carbachol. This appears to rule out EDHF-mediated responses as high extracellular K\(^+\) would prevent activation of K\(^+\) channels and therefore hyperpolarisation of smooth muscle cells.

Hydrogen peroxide has been implicated as an EDHF mediator (Beny and von der Weid, 1991) due to reports that catalase, which inhibits hydrogen peroxide production, abolishes EDHF-induced relaxations in human mesenteric arteries (Matoba et al., 2002), mice mesenteric arteries (Matoba et al., 2000) and in porcine and human coronary microvessels (Matoba et al., 2003; Miura et al., 2003). Hydrogen peroxide can be synthesised by endothelial cells (Matoba et al., 2000) and can have a direct hyperpolarising effect on vascular smooth muscle (Beny and Weid, 1991) and can cause hyperpolarisation by activating a range of K\(^+\) channels (Wei et al., 1996; Sobey et al., 1997; Barlow et al., 1998; Hayabuchi et al., 1998) and by inducing the release of vasodilator prostacyclin via COX-1 enzymes (Thengchaisri et al., 2003). Interestingly, Chaytor et al. (2003) demonstrated that hydrogen peroxide causes relaxation in the rabbit femoral artery and it was concluded that hydrogen peroxide can be characterised as a relaxing factor distinct from hyperpolarising mechanisms. In small mesenteric arteries hydrogen peroxide mediates vasorelaxation elicited by anandamide and N-oleylethanolamine and the enhanced EDHF-type responses in smaller mesenteric arteries (Wheal and Randall, 2012). However, in the present study catalase had no effect on the residual relaxation produced by carbachol in the presence of L-NAME. Thus, hydrogen peroxide is not acting as an EDHF or a modulator of EDHF in the aorta of the SHR.

Gap-junctions connecting endothelial cells and smooth muscle cells are thought to play a role in propagating endothelium-dependent vascular responses. Gap junctions are comprised of connexin
proteins (Hill et al., 2002; Segretain et al., 2004) and can allow the movement of ions and messengers between cells to elicit vascular actions. Specific connexin knock-out animal models, including connexin40, are characterised by decreased endothelium-dependent vasodilatation (de Wit et al., 2003). However, the residual relaxation to carbachol in SHR aortae was insensitive to a gap-junction inhibitor, carbenoxolone. Therefore, the residual carbachol response that could be contributing to the enhanced endothelial function of SHR aortae does not involve gap-junctions.

Interestingly, in SHRs subjected to chronic activation of TRPV1 with dietary capsaicin caused an increase in TRPV1-mediated NO production and resulted in improved endothelium-dependent vasorelaxation of mesenteric arteries (Yang et al., 2010). Capsaicin pre-treatment did not affect the responses to carbachol in either WKY or SHR aortae, ruling out sensory-nerve mediated activity in endothelium-dependent relaxations. It also demonstrates that capsaicin pre-treatment does not act as a general inhibitor of vasorelaxant responses in the preparations used. It is a possibility that this residual response to carbachol is due to the liberation of stored NO in SHR aortae, while it has also been shown that certain vessels can synthesise NO via an L-arginine independent pathway (Kemp and Cocks, 1997). Any differences in endothelial function did not account for the enhanced effects of endocannabinoids observed in aortic rings from hypertensive rats reported in Chapter 3. Responses to oleamide and anandamide were robust in endothelial denuded vessels and insensitive to the presence of L-NAME.

In summary, this chapter describes increased endothelium-dependent relaxations of SHR aortae compared to those from normotensive animals. Initially, the enhanced endothelial responses appeared contradictory to much of the literature, however as discussed above this contradiction is probably due to age-related
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differences in endothelial function. Endothelium-dependent relaxations in both SHR and WKY aortae were sensitive to the presence of L-NAME, although a residual relaxant component independent of NO existed in SHR vessels. This residual relaxation was also independent of sensory nerve mediated activity, EDHF, COX-enzymes, gap junctional communication and hydrogen peroxide. Therefore, enhanced endothelial function may represent an adaptive change in response to the early stages of established hypertension in SHRs, which is followed by the accelerated loss of endothelial function with aging.
Chapter 5

Effects of oleamide and anandamide in small mesenteric arteries from spontaneously hypertensive rats
Chapter 5 Effects of oleamide and anandamide in small mesenteric arteries from spontaneously hypertensive rats

5.1 Introduction

In chapter 3 it was demonstrated that both the endocannabinoid-like substance oleamide and anandamide caused augmented vasorelaxant effects in aortae isolated from the SHR model of hypertension. The vasorelaxant effects of endocannabinoids have previously been reported to be mediated by a variety of mechanisms and exhibit tissue and species specificity (Randall et al., 2004). Cannabinoid CB₁ receptors, sensory nerve activation, nitric oxide, the COX-pathway and EDHF have all been implicated in the vascular responses to anandamide. These mechanisms of action are tissue specific, for example, sensory nerve-mediated activity has been heavily implicated in rat mesenteric arterial resistance vessel responses to anandamide (Zygmunt et al., 1999; Ralevic et al., 2000; Harris et al., 2002; O'Sullivan et al., 2004), while sensory-nerve independent mechanisms mediate rat aortic responses and those in the coronary vasculature of different species (White et al., 2001; Ford et al., 2002; O'Sullivan et al., 2004). O'Sullivan et al. (2004) described anandamide-induced vasorelaxation of mesenteric arteries that occur through different mechanisms in conduit compared to resistance vessels.

Considering the tissue-specific nature outlined above it is likely that the effects of hypertension on vasorelaxant responses to endocannabinoids may differ between arteries. Indeed, Wheal and Randall (2009) demonstrated enhanced vasorelaxant effects to anandamide in aortae from SHR rats, while anandamide was less potent in the perfused mesenteric arterial bed. In light of this it is important to investigate the effects of oleamide in the SHR in an alternative arterial preparation. In Chapter 3 it was also reported
that there was an upregulation of endothelium-dependent vasorelaxation in aortae from hypertensive rats, which is contrary to the dogma that hypertension is associated with endothelial dysfunction. There are also a number of reports demonstrating decreased endothelium-dependent relaxations in mesenteric arteries from SHRs (Jameson et al., 1993). Therefore, it was of interest to characterise these responses in another arterial preparation.

The principal aim of this study was to assess the vasorelaxant responses to oleamide and anandamide in first order mesenteric arteries from a rat model of hypertension. Endothelial function of mesenteric arteries from SHR and WKY rats was also assessed by determining endothelium-dependent relaxations to carbachol and sensory nerve activity by responses to applied capsaicin.
5.2 Methods and Materials

5.2.1 Animals

Male SHR and WKY rats (Charles River UK) (250-350g; aged 12-18 weeks) were used during this investigation. All rats used were housed at the Biomedical Services Unit, University of Nottingham with a 12h light/dark cycle and in temperature-controlled conditions.

5.2.2 Preparation of mesenteric arteries

After the rats were killed (as described in Chapter 3) the mesenteric arterial bed was removed. First generation mesenteric arteries were dissected under a light microscope and 4 segments were mounted in a wire myograph (Danish Myo Technology) in modified Krebs’-Henseleit buffer solution (NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 4.2, NaHCO₃ 25, D-glucose 10, CaCl₂ 2 (mM)) at 37°C and gassed continuously (5% CO₂/95% O₂) (O’Sullivan et al., 2004) (Figure 5.1). Vessels were equilibrated to 9.8mN of tension and subsequently contracted with KCl (60mM) to check viability of vessels. Washout was performed to return vessels to basal tone and re-equilibrated if necessary. Vessels were pre-contracted with methoxamine. Concentration-response curves were constructed for oleamide (10nM-100µM), anandamide (1nM-10µM) (Figure 5.2), capsaicin (10nM-10µM) and carbachol (10nM-10µM). Responses to anandamide were also carried out in the presence of L-NAME (300µM).
Figure 5.1 Myograph set-up. A=transducer, B=Krebs filled organ bath, C=micrometer screw, D=hook attached to micrometer screw, E=hook attached to transducer and F=tissue segment.

Figure 5.2 Representative trace of the response to anandamide in a mesenteric artery segment from a SHR rat. A=1nM, B=3nM, C=10nM, D=30nM, E=100nM, F=300nM, G=1µM, H=3µM and I=10µM.
5.2.3 Drugs and reagents

Oleamide and anandamide were obtained from Tocris Co. (UK). All other drugs used in this investigation were purchased from Sigma Chemicals Co. (UK). Anandamide and capsaicin were dissolved in ethanol at stock concentrations of 10 mM. DMSO was used to dissolve oleamide. All other drugs were dissolved in distilled water. L-NAME was incubated for approximately 20 minutes before pre-contraction of vessels with methoxamine.

5.2.4 Statistical analysis

All responses are expressed as mean percentage vasorelaxation with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0 software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation

\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log\text{EC}_{50} - X \times \text{Hillslope})}} \]

when \( X = \log \)arithm of agonist concentration and \( Y = \)response from Bottom to Top in a sigmoidal shape]. The curves were used to determine potency \((\text{pEC}_{50})\) and maximal response \((\text{R}_{\text{max}})\) values. Potency \((\text{pEC}_{50})\) is the negative log of agonist concentration that reduced methoxamine-induced contraction by 50%. The maximal response relates to the maximum percentage vasorelaxation of methoxamine-induced pre-contraction. Statistical significance was determined using two-tailed unpaired Student’s t-test between two data sets or one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. Statistical significance was determined using one-way ANOVA unless stated otherwise in the figure legend. \( P \)-values <0.05 were considered significant.
5.3 Results

5.3.1 Vasorelaxant responses to oleamide in mesenteric arteries isolated from SHR and WKY rats

Oleamide caused concentration-dependent vasorelaxation of mesenteric arteries from both SHR ($R_{\text{max}}=33.9\pm4.6\%$, $pEC_{50}=4.73\pm0.23$, $n=6$) and WKY rats ($R_{\text{max}}=30.5\pm3.8\%$, $pEC_{50}=4.85\pm0.24$, $n=6$) (Figure 5.3) and these did not differ between strains.

Figure 5.3

![Graph showing vasorelaxant responses to oleamide in mesenteric arteries isolated from WKY and SHR rats. Mean data with bars indicating S.E.M displayed. Experiments were analysed using Student’s t-test.](image-url)
5.3.2 Vasorelaxant responses to anandamide in mesenteric arteries isolated from SHR and WKY rats

Maximal vasorelaxant responses to anandamide were significantly (P<0.001) enhanced in mesenteric arteries from SHR ($R_{\text{max}}=28.1\pm3.3\%$, $n=8$ SHR) compared to WKY rats ($R_{\text{max}}=12.9\pm3.1\%$, $n=8$) (Figure 5.4). However, the potency of anandamide was similar in SHR and WKY preparations ($\text{pEC}_{50}=6.81\pm0.62$, $n=8$ WKY; $\text{pEC}_{50}=8.06\pm0.54$, $n=8$ SHR).

Figure 5.4

Figure 5.4 Vasorelaxant responses to anandamide in mesenteric arteries isolated from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
5.3.3 Effects of L-NAME on the vasorelaxant responses to anandamide in mesenteric arteries isolated from SHR and WKY rats

L-NAME did not affect the vasorelaxant response to anandamide in mesenteric arteries from SHR rats ($R_{\text{max}} = 19.8 \pm 1.9\%$, $pEC_{50} = 8.11 \pm 0.44$, $n=8$ SHR). However, anandamide did not cause vasorelaxation in the presence of L-NAME in WKY preparations (Figure 5.5).

Figure 5.5 Vasorelaxant responses to anandamide in the presence of L-NAME (300µM) in mesenteric arteries isolated from WKY and SHR rats. Mean data with bars indicating S.E.M displayed.
5.3.4 Vasorelaxant responses to carbachol in mesenteric arteries isolated from SHR and WKY rats

Carbachol caused vasorelaxation of mesenteric arteries from both SHR ($R_{\text{max}}=83.4\pm2.7\%$, $n=9$) and WKY rats ($R_{\text{max}}=81.7\pm4.2\%$, $n=9$), such that maximal responses were similar. However, carbachol was significantly ($P<0.001$) more potent in SHR arteries ($pEC_{50}=7.44\pm0.10$, $n=9$) compared to normotensive controls ($pEC_{50}=6.77\pm0.13$, $n=9$) (Figure 5.6).

Figure 5.6

![Graph showing vasorelaxant responses to carbachol in mesenteric arteries isolated from WKY and SHR rats. Mean data with bars indicating S.E.M displayed. Experiments were analysed using Student’s t-test.](image-url)
5.3.5 Vasorelaxant response to capsaicin in mesenteric arteries isolated from SHR and WKY rats

Capsaicin caused comparable maximal vasorelaxant responses in SHR ($R_{\text{max}}=40.0\pm3.4\%$, $n=9$) and WKY arterial preparations ($R_{\text{max}}=38.8\pm5.1\%$, $n=9$). However, capsaicin was significantly ($P<0.001$) more potent in mesenteric arteries from SHRs ($p\text{EC}_{50}=7.77\pm0.33$, $n=9$) compared to normotensive controls ($p\text{EC}_{50}=5.95\pm0.25$, $n=9$) (Figure 5.7).

Figure 5.7

![Figure 5.7 Vasorelaxant responses to capsaicin in mesenteric arteries isolated from WKY and SHR rats. Mean data with bars indicating S.E.M displayed. Experiments were analysed using Student’s t-test.](image-url)
Chapter 5 Effects of oleamide and anandamide in small mesenteric arteries from spontaneously hypertensive rats

5.4 Discussion

The principal finding of Chapter 3 was that the vasorelaxant responses to the endocannabinoid-like substance, oleamide are substantially enhanced in the aorta isolated from the SHR model of hypertension and that this may be related to alterations in prostanoid metabolism. Having described the enhanced responses in aortic rings from SHR rats, the vascular effects of oleamide and anandamide in a different arterial preparation was investigated. The primary finding of this chapter was that, in contrast to the enhancement of responses in aortae from SHR rats, oleamide caused comparable vasorelaxation of mesenteric arteries from both strains. This is consistent with the literature showing oleamide to be a vasodilator of rat mesenteric vasculature (Hoi and Hiley, 2006; Sudhahar et al., 2009). The results also support the tissue specific nature of oleamide-induced vasorelaxation in arteries isolated from the SHR.

Endocannabinoid-induced responses in hypertension have previously been demonstrated to be dependent on the arterial preparation used. Wheal and Randall (2009) reported differential effects of hypertension on vasorelaxation caused by anandamide in different isolated arteries. Wheal and Randall (2009) investigated anandamide-induced vasorelaxation of aortic rings and perfused mesenteric arterial beds from two different models of hypertension; the SHR model and in rats made hypertensive by chronic inhibition of NO synthase with L-NAME. In the L-NAME model of hypertension anandamide produced augmented responses in mesenteric arterial preparations, while in aortic rings vasorelaxation was comparable to normotensive controls (Tep-Areenan et al., 2002; Wheal and Randall, 2009). Furthermore, anandamide caused increased maximal relaxations in aortic rings from SHR rats, but in mesenteric
arterial beds anandamide was actually less potent in beds from SHR rats (Wheal and Randall, 2009). The literature demonstrates how the vasorelaxant effects of endocannabinoids in hypertension are dependent on the arterial bed used, possibly as a result of differential effects of established hypertension on arteries, or due to contrasting vasodilator mechanisms mediating responses in different preparations. For example, in aortae from the Wistar rat anandamide induced vasorelaxation via a Pertussis toxin-sensitive mechanism and independently of sensory-nerve mediated activity, while in perfused mesenteric arterial bed vasorelaxant responses to anandamide involved sensory nerves (Harris et al., 2002; O’Sullivan et al., 2005). Furthermore, in the larger mesenteric arteries the responses to anandamide appear also most exclusively via sensory nerves (O’Sullivan et al., 2004). Similar tissue specificity exists in oleamide-induced vascular responses. Sudhahar et al., (2009) described vasorelaxation to oleamide in small mesenteric resistance arteries from Wistar rats that involved TRPV1 and CB1 receptors. In contrast, Chapter 2 has shown TRPV1 and CB1 to be unimportant in oleamide responses in Wistar aortae. This tissue specificity could explain why the actions of oleamide are affected differently by hypertension in aortae compared to mesenteric arteries.

In the present study, it was demonstrated that anandamide elicited enhanced vasorelaxation of first order mesenteric arteries from SHR rats compared to those from normotensive WKY rats. In Chapter 3, it was discussed how anandamide similarly caused enhanced responses in aortic rings from hypertensive rats. Indeed, the magnitude of the vasorelaxant response to anandamide in aortae from SHR rats was comparable between aortic rings and mesenteric arteries, with approximately 30-40% relaxation of induced tone. This is in contrast to Wheal and Randall (2009) who showed that anandamide was less potent in smaller mesenteric arteries from
SHR rats. However, the findings of the present chapter are supported by reports in conscious SHRs, where methanandamide produced enhanced depressor responses (Li et al., 2003). The discrepancy described between the current study and Li et al., (2003) with Wheal and Randall (2009) is possibly a question of age. Li et al., (2003) used rats aged 8-10 weeks, of age. The present study used rats of approximately 12-18 weeks and it is possibly that the discrepancies surrounding anandamide-induced vasorelaxation in SHR is due to the effects of a longer period of established hypertension on the mesenteric vasculature.

The decreased potency of anandamide in mesenteric arteries from SHR rats reported elsewhere was associated with impaired NO-dependent vasorelaxation (Wheal and Randall, 2009). However, the current chapter describes increased potency of carbachol, suggesting that endothelium-dependent vasorelaxation is actually upregulated in mesenteric arteries. Previously, Randall et al. (1991) reported intact endothelium-dependent vasodilatation of mesenteric arterial beds in rats aged 12-15 weeks. Previous studies have proposed a link between hypertension and upregulated NO-dependent mechanisms in the mesenteric vasculature from SHR rats (Marin et al., 2000; Chang et al., 2002). Chang et al. (2002) investigated changes in the perfusion pressure of mesenteric arterial beds of SHR and WKY rats using an in situ mesenteric system. It was reported that endothelium-dependent vasodilatation induced by acetylcholine was enhanced in SHR rats compared to normotensive controls (Chang et al., 2002). In young SHR and WKY rats (4 weeks old) endothelium-dependent relaxations of mesenteric resistance arteries were comparable, however vasorelaxation to acetylcholine is impaired in older SHR rats (16 and 28 weeks) (Jameson et al., 1993). This chapter reports that carbachol is more potent in mesenteric arteries from SHR rats, which is consistent with
enhanced endothelium-dependent responses in hypertension (Chapter 4). As discussed in Chapter 3 this is contradictory to the established dogma that the SHR model of hypertension is associated with endothelial dysfunction, however it is argued that endothelial dysfunction in SHR rats is positively correlated with age and thus the duration of established hypertension. Furthermore, experimental and physiological conditions may affect whether endothelium-dependent relaxation of hypertensive arteries is impaired or not (Li and Bukoski, 1993). Li and Bukoski (1993) demonstrated impaired relaxations to acetylcholine of mesenteric resistance arteries from SHR rats aged 12-15 weeks. However, endothelial dysfunction was only apparent in arteries pre-contracted with noradrenaline. The authors concluded that endothelial dysfunction occurred in SHR rats due to increased endothelium-derived contracting factor (EDCF) elicited during pre-contraction by noradrenaline. Endothelium-dependent relaxations were equal in arteries from both hypertensive and normotensive strains after pre-contraction with vasopressin and in the presence of indomethacin in noradrenaline contracted vessels (Li and Bukoski, 1993). However, it is also possible that vasopressin was causing the release of endothelium-derived relaxing factors resulting in normalised endothelium-dependent relaxation between strains and so masking differences in endothelial function (Katusic et al., 1984; Randall et al., 1988; Suzuki et al., 1989). Indeed, in the rat isolated superior mesenteric arterial bed responses to vasopressin were modulated by an endothelium-derived relaxing factor (Randall et al., 1988). In human mesenteric arteries, vasopressin caused the release of endothelium-derived vasodilator prostaglandins (Martinez et al., 1994). Therefore, while agonist choice for pre-contraction may affect endothelial-dysfunction, it is possible that vasopressin, through the release of endothelial factors, masks any underlying differences in endothelial function. Moreover, differences in
endothelial function have also been described in vessels pre-
contracted with serotonin and prostaglandin (Luscher and Vanhoutte,
1986; Tesfamariam and Halpern, 1988). In addition, Vindis et al.,
(2009) reported endothelial dysfunction in third order mesenteric
arteries from SHR rats in experiments where concentrations of
noradrenaline were used that elicited the same level of contraction
in vessels form both hypertensive and normotensive strains.

In the present chapter, it was demonstrated that capsaicin is a more
potent vasorelaxant in mesenteric arteries from SHR rats. In
Chapter 3 it was described how in aortic segments capsaicin-induced
responses were equal between the strains. This suggests that in
mesenteric vasculature there may be alterations in sensory-nerve
mediated activity in hypertension but not in the aortic. In addition
an alternative mechanism of action of capsaicin-induced
vasorelaxation has been identified in Chapter 5. Li et al. (2003)
reported enhanced hypotensive effects of methanandamide and
capsaicin in SHR rats, which were sensitive to the presence of
capsazepine. The authors proposed that sensory-nerve activity was
upregulated in hypertension. Wheal and Randall (2009) reported
that in mesenteric arterial beds from the L-NAME-induced model of
hypertension capsaicin caused enhanced vasorelaxation in a manner
sensitive to capsaicin pre-treatment. The increased sensory-nerve
mediated activity associated with mesenteric beds from
hypertension was responsible for augmented vasorelaxation to
anandamide (Wheal and Randall, 2009). Furthermore, the authors
described comparable vasorelaxation to anandamide in aortae from
L-NAME-induced hypertensive and normotensive rats, which
correlated with comparable sensory nerve mediated activity in
aortae from hypertensive and normotensive rats. It was also
demonstrated that the mesenteric vasculature of the SHR was
associated with increased expression of the CGRP receptor (Li et al.,
2003). It is possible that mesenteric arteries from SHR rats are more sensitive to capsaicin, due to the release of the neuropeptide CGRP and its effects at the upregulated CGRP receptor.

Enhanced blood pressure decreases to both anandamide and capsaicin in rats made hypertensive by a high-salt diet are blocked by capsazepine (Wang et al., 2005). In addition, Wang and Wang (2007) reported that methanandamide caused greater release of CGRP in high-salt hypertensive rats compared to normotensive controls and that mesenteric expression of CGRP receptor was upregulated. Interestingly, hypertension elicited by high salt resulted in augmented production of anandamide (Wang et al., 2007). Therefore, in the high-salt model of hypertension, increased anandamide production acting at upregulated sensory-nerve mediated mechanisms may be an adaptive change to regulate high blood pressure. This regulation or blunting of hypertension through sensory-nerve mediated pathways is further supported by the fact that capsazepine caused enhanced pressor responses in hypertensive rats compared to normotensive controls (Wang and Wang, 2007). Similarly, this chapter reports enhanced sensory-nerve mediated activity in mesenteric arteries from SHR rats that correlates with augmented anandamide-induced vasorelaxation. Therefore, augmented sensory-nerve activity could underpin the increased vasorelaxation to anandamide in mesenteric arteries of SHR rats and may represent a physiological limit on blood pressure increases. In contrast, oleamide-induced responses are comparable between SHR and WKY mesenteric arteries and so are unaffected by the differential sensory-nerve mediated activity. This suggests that vasorelaxation to oleamide of mesenteric arteries from WKY and SHR functions independently of sensory nerves.

Interestingly, in SHRs subjected to chronic activation of TRPV1 with dietary capsaicin mean arterial pressure was lowered (Yang et al.,
2010). This was associated with increases in nitrite levels, an indication of enhanced NO production, and it was also demonstrated that dietary capsaicin improved endothelial function of mesenteric arteries from SHRs (Yang et al., 2010). Thus, the authors concluded that long-term activation of TRPV1 restored endothelial function through upregulated NO release, which was supported by observations in a mouse model. Chronic activation of TRPV1 in mice augmented eNOS levels in endothelial cells and mesenteric arteries, and this effect was absent in TRPV1 knockout mice (Yang et al., 2010). In the high salt model of hypertension, Wang et al. (2007) described enhanced production of anandamide. Taken together it is therefore possible that increased plasma levels of anandamide in response to hypertension and possible increases in TRPV1 expression could result in augmented long-term activation of these receptors in hypertensive animals. This presents a theoretical mechanism for improved endothelial function in arteries from younger SHR rats.

In summary, this chapter demonstrates that oleamide caused comparable vasorelaxant responses in mesenteric arteries from hypertensive and normotensive rats. This is in contrast to oleamide-induced vasorelaxation of aortic segments, which was augmented in hypertension. This interestingly illustrates a tissue specific nature of the effects of hypertension on oleamide-induced vasorelaxation. However, anandamide elicited enhanced vasorelaxant responses in both aortae and mesenteric arteries from SHR rats in comparison to normotensive controls. The augmented responses to anandamide in mesenteric arteries were not due to differences in NO-dependent mechanisms. Mesenteric arteries from SHR rats demonstrated increased sensitivity to capsaicin, possibly due to alterations in sensory-nerve mediated mechanisms.
Chapter 6

Vasorelaxation to capsaicin and its effects on calcium influx in arteries
6.1 Introduction

Capsaicin is the active component of hot chilli peppers and has been shown to induce a range of pharmacological effects by activating the TRPV1 receptor (Caterina et al., 1997; Gunthorpe et al., 2002; Szallasi et al., 2006). In addition, chronic capsaicin exposure is a commonly used pharmacological tool for desensitising perivascular sensory nerves by exhausting neuropeptides stores.

TRPV1 is a non-selective cation channel (Caterina et al., 1997) which regulates permeability to Ca\(^{2+}\) and can be activated by a range of endogenous agonists including vanilloids (for review; Sterner and Szallasi, 1999), endocannabinoids (Zygumnut et al., 1999; Smart et al., 2000), lipoxygenase products (Hwang et al., 2000) as well as physical stimuli. The activation of TRPV1 receptors by agonists causes an increase in intracellular Ca\(^{2+}\) which results in the release of neuropeptides such as calcitonin gene related-peptide (CGRP), substance P and tachykinins (Saria et al., 1986; Franco-Cereceda et al., 1987; Geppetti et al., 1988; Mayer et al., 1990; Patacchini et al., 1999; Dunn et al., 2003). These neuropeptides can regulate vascular smooth muscle, for example CGRP causes hyperpolarisation of smooth muscle by activating K\(^+\) channels (Dunn et al., 2003).

Capsaicin and its derivatives have been shown to induce blood pressure decreases in rats (Lo et al., 2003; Li et al., 2003; Li and Wang, 2003; Wang and Wang et al., 2007). Also, the chronic activation of TRPV1 in SHRs through dietary capsaicin decreased arterial blood pressure (Yang et al., 2010). The vasodilator nature of capsaicin has been demonstrated in a number of isolated blood
vessels and the release of vasoactive neuropeptides has been implicated in capsaicin-induced vasorelaxation (Franco-Cereceda et al., 1987; Franco-Cereceda and Rudehill, 1989; Jansen et al., 1990; Holzer, 1992; Li and Wang, 2003). Pre-treatment of cerebral arteries, dura matter and primary sensory nerves with capsaicin depleted stores of substance P, Neurokinin A and CGRP, hence the use of capsaicin as a pharmacological tool to desensitise sensory nerves (Gamse et al., 1981; Saito and Goto, 1986; Dux et al., 2003).

Studies have also demonstrated the involvement of nitric oxide (NO) in capsaicin-induced responses. In the perfused coronary circulation of rabbits, capsaicin elicited relaxations that were sensitive to both NOS inhibition and CGRP-receptor inhibition (Mitchell et al., 1995). Yang et al., (2010) demonstrated that chronic activation of TRPV1 by dietary capsaicin caused increased activity of endothelial NOS in mouse mesenteric arteries. The effects of capsaicin were absent in TRPV1 knockout mice demonstrating the involvement of these receptors. The authors proposed that long-term TRPV1 activation on endothelial cells resulted in calcium influx causing the phosphorylation of protein kinase A (PKA) and subsequent activation of NOS (Yang et al., 2010).

Previous chapters have shown that a 1h pre-treatment of aortae from Wistar rats with capsaicin abolishes responses to oleamide. Moreover, capsaicin pre-treatment reduces the enhanced vasorelaxation of SHR aortae by anandamide and oleamide to levels produced in normotensive vessels. Taken on its own this suggests that oleamide and anandamide responses are driven by sensory-nerve mediated activity. However, the vasorelaxant responses to oleamide and anandamide were insensitive to the TRPV1 antagonist, capsazepine, and the cation channel blocker ruthenium red. Thus, anandamide and oleamide are exerting effects independently of
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TRPV1 but in a manner sensitive to capsaicin pre-treatment. This would suggest that capsaicin can function by mechanisms that are distinct from sensory-nerve activity to cause vasorelaxation and affect the vasorelaxant responses to oleamide and anandamide. According to the literature this could be mediated by the direct action of capsaicin on ion channels. It is therefore necessary to investigate further the mechanisms of actions involved in the vascular effects of capsaicin.

Recent studies have demonstrated that capsaicin can also affect vascular control by mechanisms distinct from activation of TRPV1 on sensory nerves. In rat aortic smooth muscle cells the inhibition of L-type Ca\textsuperscript{2+} channels was identified as the mechanism underlying capsaicin-induced relaxations (Lo et al., 1995). In addition, potassium channel activation has also been implicated (Ellis et al., 1997; Zhu et al., 1997; Yeon et al., 2001; Fujimoto et al., 2006). Also involved in the capsaicin relaxation is COX-2. In vivo experiments involving human mucosal vascular beds have demonstrated that 10µM capsaicin elicits vasodilatation which is insensitive to capsazepine and the CGRP receptor antagonist CGRP8-37 (Van Crombruggen et al., 2011). The vascular response to capsaicin was attenuated by an EP\textsubscript{1} prostanoid receptor antagonist and by inhibition of COX-2 with NS398, indicating capsaicin decreased the production of the vasoconstrictor prostanoids. Therefore, authors concluded that capsaicin induced TRPV1-independent vasodilatation by reducing production of PGE\textsubscript{2} via COX-2 activity. The endocannabinoid system has been implicated in regulating capsaicin-induced bronchospasm in guinea pigs (Calignano et al., 2000).

In light of the involvement of TRPV1-independent mechanisms in capsaicin-induced vasorelaxation, and capsaicin’s importance as a pharmacological tool in desensitising perivascular sensory nerves,
this chapter aimed to characterise the vascular actions of capsaicin in isolated arteries. In particular, the roles of TRPV1 receptors and the effects of calcium influx in capsaicin-induced vasorelaxation of rat isolated aortae and porcine coronary arteries were examined.
6.2 Methods and Materials

6.2.1 Animals

Male Wistar rats were killed and aortae removed and set-up following the protocols outlined in Chapter 2. Porcine hearts were obtained from a local abattoir and transported in ice-cold Krebs’-Henseleit buffer solution (NaCl 118, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 4.2, NaHCO₃ 25, D-glucose 10, CaCl₂ 2 (mM)). Proximal coronary arteries were removed from the hearts and stored in a refrigerator at approximately 4°C in pre-gassed Krebs’-Henseleit buffer solution having been cleaned thoroughly of connective tissue by blunt dissection and cut into segments of 3-5mm.

6.2.2 The vascular response to capsaicin in aortic rings and porcine coronary artery

Aortic rings and porcine coronary arteries were placed onto two metal wires, with one being fixed and the other attached via thread to an isometric transducer measuring tension and placed into 50ml organ baths. The organ baths were filled with modified Krebs’-Henseleit buffer solution at 37°C and gassed steadily (5% CO₂/95% O₂). The aortic rings were allowed to equilibrate to 9.8mN of tension and coronary arteries were equilibrated to 49mN. Tension was measured by a Leitica force transducer coupled to an ADInstruments MacLab recording system.

Aortic vessels were pre-contracted with the α₁-adrenoceptor agonist methoxamine (10µM) prior to the addition of capsaicin (1nM-10µM), which was added cumulatively at 5 minute intervals to construct concentration-response curves. Porcine coronary arteries were pre-contracted with U46619 (1-70nM), a thromboxane mimetic, prior to the construction of capsaicin concentration-response curves (1nM-100µM) (Figure 6.1) to achieve a submaximal contraction of 50-
80% of maximal KCl (60mM) response. To investigate the role of sensory nerves, some vessels were subjected to pre-treatment with 10µM capsaicin for 1h (Zygmunt et al., 1999). Capsaicin pre-treatment was followed by a 20min wash-out period, prior to pre-contraction. In aortic rings, concentration-responses curves to capsaicin were also constructed in the presence of capsazepine (1µM), a TRPV1 antagonist (White et al., 2001) and ruthenium red (10µM), a cation channel blocker (Harris et al., 2002).

Figure 6.1

Figure 6.1 Representative trace of the response to capsaicin in the porcine coronary artery. A=1nM, B=3nM, C=10nM, D=30nM, E=100nM, F=300nM, G=1µM, H=3µM, I=10µM, J=30µM and K=100µM.

6.2.3 The effect of capsaicin on contractile responses to calcium re-introduction

Aortae were equilibrated to 9.8mN and porcine coronary arteries to 49mN for 1h in Ca²⁺ free Krebs-Henseleit buffer. After equilibration in Ca²⁺-free, high K⁺ buffer (NaCl 62.5, KCl 59.4, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, D-glucose 10 (mM)) capsaicin (either 1 µM, 3 µM or 10 µM) was added to vessels with others being used as controls. Concentration-response curves for the re-introduction of calcium (10nM-3mM) (Figure 6.2) were constructed to test the effects of
capsaicin on calcium-influx induced contraction. Some experiments were performed in the presence of capsazepine (10µM) to assess the potential involvement of TRPV1 receptors. Other experiments were carried out in the presence of oleamide (10µM) and anandamide (10µM) in order to characterise the effects of endocannabinoids on calcium influx. Concentration-response curves were also constructed in porcine coronary arteries to Bay-K 8644 (1nM-3µM), an L-type Ca\(^{2+}\) channel agonist. In preliminary experiments Bay-K 8644 did not produce a vasoconstrictor effect in the rat isolated aorta.

Figure 6.2

![Figure 6.2](image)

Figure 6.2 Representative trace of the contractile response to calcium in the porcine coronary artery. A=10nM, B=30nM, C=100nM, D=300nM, E=1µM, F=3µM, G=10µM, H=30µM, I=100µM, J=300µM, K=1mM and L=3mM.

6.2.4 Drugs and reagents

Capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide), capsazepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), methoxamine hydrochloride (α-(1-Aminoethyl)-2,5-dimethoxybenzyl alcohol hydrochloride), U46619 (9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F\(_{2α}\),
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ruthenium red (ammoniated ruthenium oxychloride) and Bay-K 8644 (1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid, methyl ester) were purchased from Sigma Chemicals Co. (UK). Oleamide (cis-9-Octadecenoamide) and anandamide (arachidonylethanolamide) were purchased from Tocris Co. (UK). Capsaicin, capsazepine, anandamide and Bay-K 8644 were dissolved in ethanol at stock concentrations of 10mM. Methoxamine hydrochloride and ruthenium red were dissolved in water at a stock concentration of 100mM and 10mM. U46619 and oleamide were dissolved in DMSO (Dimethyl sulfoxide). Capsazepine was added 20 minutes prior to the addition of contractile agents. Ruthenium red was present 30 minutes before pre-contraction.

6.2.5 Statistical analysis

All responses are expressed as mean percentage vasorelaxation with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0 software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation: 

\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1+10^{(\text{LogEC}_{50} - X) \cdot \text{Hillslope}})}, \]

when \( X = \) logarithm of agonist concentration and \( Y = \) response from Bottom to Top in a sigmoidal shape. The curves were used to determine potency (pEC\textsubscript{50}) and maximal response (R\textsubscript{max}) values. Potency (pEC\textsubscript{50}) is the negative log of agonist concentration that reduced methoxamine-induced contraction by 50%. The maximal response relates to the maximum percentage vasorelaxation of methoxamine-induced pre-contraction. Statistical significance was determined using two-tailed unpaired Student’s t-test between two data sets or one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. Statistical significance was determined using one-way
ANOVA unless stated otherwise in the figure legend. P-values <0.05 were considered significant.
6.3 Results

6.3.1 Vascular responses to capsaicin in rat isolated aortae

Capsaicin caused concentration-dependent vasorelaxation of the aortic rings ($R_{\text{max}}=25.1\pm3.3\%$, $pEC_{50}=6.29\pm0.26$, $n=7$), which was significantly ($P<0.001$) greater than the small vasorelaxant response caused by the vehicle control (0.15% of final bath volume) ($R_{\text{max}}=5.5\pm1.4\%$, $n=7$) alone (Figure 6.3).

Figure 6.3

Figure 6.3 Vasorelaxant responses to capsaicin and the vehicle control (ethanol) in aortic rings pre-contracted with methoxamine from Wistar rats. Mean data with bars indicating S.E.M are displayed.
6.3.2 Effects of capsaicin pre-treatment on capsaicin-induced vasorelaxation of rat isolated aortae

Capsaicin-induced vasorelaxation of the rat aorta was unaffected by a 1h capsaicin (10µM) pre-treatment ($R_{\text{max}}=27.4\pm1.9\%$, $\text{pEC}_{50}=6.42\pm0.14$, n=7) (Figure 6.4).

Figure 6.4

Figure 6.4 Vasorelaxant responses to capsaicin in rat isolated aortae pre-contracted with methoxamine after a 1h capsaicin (10µM) pre-treatment. Mean data with bars indicating S.E.M are displayed.
6.3.3 Involvement of TRPV1 receptors in capsaicin-induced vasorelaxation of rat isolated aortae

The vasorelaxant responses to capsaicin ($R_{\text{max}}=30.4\pm2.8\%$, $pEC_{50}=6.57\pm0.25$, $n=8$) were also unaffected by the presence of capsazepine (1µM) ($R_{\text{max}}=32.6\pm3.2\%$, $pEC_{50}=6.44\pm0.24$, $n=8$) (Figure 6.5).

Figure 6.5

![Graph showing vasorelaxant responses to capsaicin in rat isolated aortae pre-contracted with methoxamine in the presence of capsazepine (1µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.](image-url)
6.3.4 Effects of ruthenium red on vasorelaxant responses to capsaicin in the rat isolated aorta

Similarly, responses to capsaicin ($R_{\text{max}}=21.2\pm2.6\%$, $pEC_{50}=6.1\pm0.20$, $n=6$) were unaffected by 10µM ruthenium red ($R_{\text{max}}=28.0\pm7.5\%$, $pEC_{50}=5.85\pm0.37$, $n=6$) (Figure 6.6).

Figure 6.6

Figure 6.6 Vasorelaxant responses to capsaicin in rat isolated aortae pre-contracted with methoxamine in the presence of ruthenium red. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
6.3.5 Effects of capsaicin on the contractile responses to calcium re-introduction in rat isolated aortae

The re-introduction of calcium into a calcium-free, high potassium buffer caused concentration-dependent contraction of the rat aortic rings (Figure 6.7). The contractile response caused by calcium re-introduction was significantly (P<0.001) reduced in the presence of 30µM capsaicin, such that at 3mM calcium the increase in tone was 5.6±1.0mN (n=12) compared to a control response of 15.6±2.4mN (n=10) in its absence (Figure 6.7).

Figure 6.7

Figure 6.7 Contractile responses in aortic rings from Wistar rats to Ca$^{2+}$ re-introduction in a Ca$^{2+}$ free, high K$^+$ buffer in the presence of 30µM capsaicin. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
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6.3.6 Vascular effects of capsaicin in the porcine isolated coronary artery

Capsaicin caused a concentration-dependent vasorelaxation of porcine coronary arteries that was significantly ($P<0.05$) greater than responses to the vehicle control (ethanol), such that at 100µM capsaicin the relaxation was $98.5\pm9.9\%$ ($n=6$) compared to a vehicle response of $49.1\pm13.8\%$ ($n=6$) (Figure 6.8).

Figure 6.8

![Graph showing vasorelaxant responses to capsaicin and the vehicle control (ethanol) in porcine isolated coronary arteries pre-contracted with U46619. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.](image-url)
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6.3.7 Involvement of TRPV1 receptors in capsaicin-induced vasorelaxation of porcine isolated coronary artery

Vasorelaxant responses to capsaicin in the porcine coronary artery were unaffected by the presence of the TRPV1 receptor antagonist capsazepine (10µM). At 100µM capsaicin the maximal relaxation was 115 ± 5% (n=6) compared to 108±7% (n=6) in the presence of capsazepine (10µM) (Figure 6.9).

Figure 6.9

![Graph showing vasorelaxant responses to capsaicin in porcine coronary artery pre-contracted with U46619 in the presence of capsazepine (10µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.](image)

Figure 6.9 Vasorelaxant responses to capsaicin in porcine coronary artery pre-contracted with U46619 in the presence of capsazepine (10µM). Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
6.3.8 Effects of capsaicin on the contractile response to calcium re-introduction in porcine isolated coronary artery

The re-introduction of Ca$^{2+}$ into a Ca$^{2+}$ free, high K$^+$ environment caused contraction of porcine coronary arterial segments ($R_{\text{max}}=110.1\pm5.6\text{mN}$, $pEC_{50}=3.88\pm0.10$, $n=6$) (Figure 6.10). The contractile responses remained intact in the presence of 1µM ($R_{\text{max}}=100.9\pm4.5\text{mN}$, $pEC_{50}=3.91\pm0.09$, $n=6$) and 10µM capsaicin ($R_{\text{max}}=106.0\pm5.9\text{mN}$, $pEC_{50}=3.64\pm0.09$, $n=6$) (Figure 6.10). However, the presence of 30µM capsaicin caused a significant ($P<0.0001$) decrease in both the maximal contractile response ($R_{\text{max}}=46.5\pm5.8\text{mN}$, $n=6$) and its potency ($pEC_{50}=3.21\pm0.15$, $n=6$) ($P<0.01$) (Figure 6.10).
Figure 6.10 Contractile responses in segments of porcine coronary artery to Ca\textsuperscript{2+} re-introduction in a Ca\textsuperscript{2+} free, high K\textsuperscript{+} buffer in the presence of 1µM, 10µM and 30µM capsaicin. Mean data with bars indicating S.E.M are displayed.

6.3.9 Effects of 100µM capsaicin and the presence of vehicle on the contractile response to calcium re-introduction in porcine isolated coronary artery

The maximal contractile response (P<0.001) and potency (P<0.01) of responses to Ca\textsuperscript{2+} re-introduction in the presence of 30µM (R\textsubscript{max}=69.6±7.0mN, pEC\textsubscript{50}=3.12±0.13, n=6) were both significantly reduced compared to the contractile responses in the presence of the vehicle control (R\textsubscript{max}=131.7±8.1mN, pEC\textsubscript{50}=3.85±0.12, n=6) (Figure 6.11). In the presence of 100µM capsaicin the contractile response to Ca\textsuperscript{2+} was completely abolished (n=5) (Figure 6.11).
Figure 6.11 Contractile responses in segments of porcine coronary artery to Ca\(^{2+}\) re-introduction in a Ca\(^{2+}\) free, high K\(^+\) buffer in the presence of vehicle control, 30µM and 100µM capsaicin. Mean data with bars indicating S.E.M are displayed.

6.3.10 Involvement of TRPV1 receptors in the effects of capsaicin on the contractile responses to calcium re-introduction

The contractile responses to Ca\(^{2+}\) re-introduction were abolished by the presence of 30µM capsaicin and 10µM capsazepine. In the presence of 30µM capsaicin alone calcium re-introduction caused a small maximal contractile response, such that the maximal response to 3mM Ca\(^{2+}\) was 17.7±4.9mN (n=6) (Figure 6.12).
Figure 6.12

Figure 6.12 Contractile responses in segments of porcine coronary artery to Ca\(^{2+}\) re-introduction in a Ca\(^{2+}\) free, high K\(^{+}\) buffer in the presence of 30µM capsaicin and 10µM capsazepine. Mean data with bars indicating S.E.M are displayed.

6.3.11 Effects of capsaicin on contractile responses to the L-type calcium channel activator Bay-K 8644

The L-type calcium channel activator caused concentration-dependent vasoconstriction of porcine isolated coronary arteries (R\(_{\text{max}}\)=60.0±3.9mN, pEC\(_{50}\)=7.15±0.16, n=9) (Figure 6.13). The vasoconstrictor responses to Bay-K 8644 were unaffected by the presence of either 1µM (R\(_{\text{max}}\)=65.0±4.8mN, pEC\(_{50}\)=7.27±0.19, n=6) or 3µM capsaicin (R\(_{\text{max}}\)=42.1±11.9mN, pEC\(_{50}\)=6.57±0.49, n=6) (Figure 6.13). However, in the presence of 10µM capsaicin the
maximal contractile response to Bay-K 8644 was significantly (P<0.05) reduced ($R_{\text{max}}=25.8\pm5.0\text{mN}$, $\text{pEC}_{50}=6.52\pm0.33$, $n=6$) (Figure 6.13). Vasoconstrictor responses to Bay-K 8644 were completely abolished in the presence of $30\mu\text{M}$ capsaicin ($n=6$) (Figure 6.13).

Figure 6.13

![Contractile responses of the porcine isolated coronary arteries to the L-type calcium channel activator Bay-K 8644 in presence of 1µM, 3µM, 10µM and 30µM capsaicin. Mean data with bars indicating S.E.M are displayed.](image-url)
6.3.12 Effects of oleamide on the contractile response to calcium re-introduction in porcine isolated coronary artery

The contractile response to Ca\(^{2+}\) re-introduction \((R_{\text{max}}=12.3\pm0.8\text{mN}, \quad \text{pEC}_{50}=3.57\pm0.09, \quad n=8)\) was unaffected by 10µM oleamide \((R_{\text{max}}=14.7\pm1.4, \quad \text{pEC}_{50}=3.54\pm0.14, \quad n=6)\) (Figure 6.14).

*Figure 6.14*

![Graph showing contractile response in segments of porcine coronary artery to Ca\(^{2+}\) re-introduction in a Ca\(^{2+}\) free, high K\(^{+}\) buffer in the presence of 10µM oleamide. Mean data with bars indicating S.E.M are displayed.](image-url)
6.3.13 Effects of anandamide on the contractile response to calcium re-introduction in porcine isolated coronary artery

The contractile responses to Ca\(^{2+}\) re-introduction ($R_{\text{max}}=12.3\pm0.8\text{mN}$, $pEC_{50}=3.57\pm0.09$, $n=8$) were unaffected by 10µM anandamide ($R_{\text{max}}=10.8\pm0.7\text{mN}$, $pEC_{50}=3.58\pm0.09$, $n=8$) (Figure 6.15).

Figure 6.15

Figure 6.15 Contractile responses in segments of porcine coronary artery to Ca\(^{2+}\) re-introduction in a Ca\(^{2+}\) free, high K\(^{+}\) buffer in the presence of 10µM anandamide. Mean data with bars indicating S.E.M are displayed.
6.4 Discussion

A major finding of this investigation is that capsaicin induces concentration-dependent vasorelaxation of the rat isolated aorta through mechanisms distinct from TRPV1 receptors and, by implication, sensory nerves. In addition, the results suggest an alternative mechanism of action for capsaicin that could drive relaxant responses in both rat aortae and porcine coronary arteries.

Capsaicin caused concentration-dependent vasorelaxation of both rat aortae and porcine coronary arteries. The relaxant responses in rat aortae were unaffected by the TRPV1 receptor antagonist capsazepine. Similarly, the presence of the cation-channel blocker ruthenium red had no effect on vascular responses to capsaicin. Pre-treatment of aortae with capsaicin, which is designed to deplete perivascular sensory nerves of vasoactive neuropeptides (Zygmunt et al., 1999), did not alter the vasorelaxation to capsaicin. Furthermore, the vasorelaxant responses to capsaicin were insensitive to both capsazepine and ruthenium red. These findings suggest that capsaicin-induced vasorelaxation of the rat aorta and porcine coronary artery does not involve TRPV1 receptors. These findings are consistent with other investigations demonstrating non-TRPV1 mechanisms of action for capsaicin. Expression of TRPV1 has previously been detected in aortic smooth muscle cells from male Wistar rats (Yang et al., 2006). Vanilloid receptor activation on vascular smooth muscle is more likely to induce vasoconstriction, whereas activation of endothelial expression receptors is likely to elicit vasorelaxation (reviewed in Firth et al., 2007). For example, endocannabinoid-induced TRPV1-mediated relaxation of the rat mesenteric bed is correlated with endothelial TRPV1 expression (Poblete et al., 2005).
The findings of a capsaicin-induced vasorelaxation which is insensitive to TRPV1 antagonism has been reported elsewhere in the literature. Gupta et al. (2007) described capsaicin-induced vasorelaxation in both human and porcine coronary arteries which was similarly insensitive to both capsazepine and ruthenium red. In addition, responses were unaffected by CGRP receptor blockade (Gupta et al., 2007). However, in contrast Bratz et al. (2008) described a capsaicin-induced relaxation of porcine coronary arteries that was partially inhibited by capsazepine. This study examined an impaired vasorelaxant response to capsaicin in a model of the metabolic syndrome, which was associated with a decreased expression of TRPV1 expression in vasculature from the metabolic syndrome model (Bratz et al., 2008).

Several other studies have also demonstrated non-sensory nerve mediated relaxations to capsaicin in rabbit coronary arteries, guinea pig ileum, equine smooth muscle and human nasal vasculature (Yeon et al., 2001; Zhu et al., 1997; Fujimoto et al., 2006; Van Crombruggen et al., 2011). Although, Gupta et al. (2007) described TRPV1-independent relaxation of human and porcine coronary arteries, they were unable to elucidate the specific mechanisms underlying the responses to capsaicin. In addition to being TRPV1-independent, responses were distinct from CGRP, NO, K+ channels, cAMP and calcium channels (Gupta et al., 2007).

Previous studies have shown that capsaicin inhibits calcium entry via channels in the rat aorta. Monsereenusorn and Kongsamut (1985) described a dose-dependent inhibition of radioactive calcium-45 uptake by capsaicin in cultured smooth muscle cells from rat aorta. To build on this Lo et al. (1995) demonstrated that capsaicin inhibited L-type Ca^{2+} channels in cultured rat aortic smooth muscle. These results suggested that L-type Ca^{2+} channel inhibition may be involved in the vascular actions of capsaicin in the rat aorta. In
support of this, the present study shows that the presence of capsaicin inhibits the contractile response caused by calcium re-introduction in aortae in a high K⁺ (60mM) calcium-free buffer, consistent with interference with calcium influx. Therefore, capsaicin-induced vasorelaxation of rat aorta and porcine coronary arteries are mediated via inhibition of calcium influx.

In the present study, capsaicin produced concentration-dependent vasorelaxant responses in porcine isolated coronary arteries. The magnitude of the vasorelaxant response was comparable to capsaicin-induced relaxations reported elsewhere (Gupta et al., 2007). Capsaicin-induced vasorelaxation of porcine coronary arteries was insensitive to capsazepine (10µM). This clearly demonstrates that vasorelaxation of coronary arteries and rat aortae in response to capsaicin are mediated independently of TRPV1 receptors. This is in accordance with Gupta et al. (2007), who described capsazepine-insensitive vasorelaxation of human and porcine coronary arteries. In contrast, Bratz et al. (2008) reported capsaicin driven vasorelaxation of porcine coronary arteries that was partially mediated by TRPV1 receptors, in an endothelial-dependent mechanism, together with nitric oxide and K⁺ channels. The differences in the literature could reflect differences in the strain of swine used. Bratz et al. 2008 examined capsaicin-induced responses in coronary arteries from obese male Ossabaw miniature swine compared to lean controls. They described an impaired response to capsaicin in coronaries from obese swine compared to lean controls, which was associated with a decreased expression of TRPV1. The capsaicin-induced relaxation of coronary arteries from lean pigs was normalised by capsazepine to that of arteries from obese swine (Bratz et al., 2008). However, there was still a large residual response to capsaicin in arteries from lean pigs in the presence of TRPV1 antagonist. The vasorelaxant effects of capsaicin
in lean coronary arteries was sensitive to L-NAME, iberiotoxin and also TEA, implicating both NO and K+ channels (Bratz et al., 2008). After all treatments a large residual relaxant effect remained and it was unclear if the involvement of NO and K+ channels may be mediated by the upstream activation of TRPV1 receptors. Bratz et al. 2008, using Fura2 imaging, demonstrated calcium-influx via TRPV1 receptors in response to capsaicin in endothelial cells from coronary arteries. Elsewhere, it has been shown that an increase in intracellular Ca2+ through TRPV1 activation by capsaicin results in upregulated NOS activity via phosphorylation by PKA (Yang et al., 2010). It is also possible that TRPV1-induced Ca2+ influx in the endothelial cells mediated the release of EDHF, although this is less likely in larger conduit vessels. Alternatively, several studies have described the direct actions of capsaicin at K+ channels on smooth muscle (Zhu et al., 1997; Yeon et al., 2001; Fujimoto et al., 2006).

In light of capsaicin-induced, capsazepine insensitive relaxation of the porcine coronary artery it was necessary to characterise the role of calcium channels in this preparation. The contractile responses induced by calcium re-introduction was significantly reduced by the presence of 30µM capsaicin and completely abolished by the presence of 100µM capsaicin. These results demonstrate that capsaicin is interfering with calcium entry into smooth muscle. One hypothesis is that activation of TRPV1 results in the downstream inhibition of L-type Ca2+ channels and so mediates decreased calcium entry. Indeed, it has previously been reported that CGRP can inhibit calcium currents in rat paracervical ganglion neurons and also modify L-type Ca2+ channel currents in the smooth muscle of rat vas deferens (Nakazawa et al., 1992; Cohen et al., 1996). However, the inhibitory effects of capsaicin on calcium influx were not inhibited by the presence of a TRPV1 competitive antagonist, indicated that it is not coupled to TRPV1 receptor activation.
Capsaicin has previously been reported to block calcium-uptake in smooth muscle cells from rat aorta (Monsereenusorn and Kongsamut, 1985; Lo et al., 1995).

Having demonstrated that capsaicin inhibits calcium-influx into vascular smooth muscle the effects of capsaicin on L-type Ca\(^{2+}\) channels were investigated. Bay-K 8644 (Bay-K) is an L-type Ca\(^{2+}\) channel activator that increases Ca\(^{2+}\)-influx (Takasu et al., 1987; Triggle and Rampe, 1989) and caused a concentration-dependent vasoconstriction of porcine coronary arteries. The presence of 10µM capsaicin significantly inhibited the maximal contractile response caused by Bay-K 8644. Interestingly, the presence of 30µM capsaicin completely abolished the contractile response to Bay-K 8644. These results suggest that capsaicin can directly inhibit L-type Ca\(^{2+}\) channel activation on vascular smooth muscle. The concentrations of capsaicin used to block the effects of Bay-K are within the range that causes capsaicin-induced vasorelaxation. Thus, it is possible that the direct inhibition of L-type Ca\(^{2+}\) channels is a mechanism of action driving vascular responses to capsaicin. This is consistent with the involvement of Ca\(^{2+}\) channels in the relaxant effect of capsaicin in smooth muscle from rat aorta (Lo et al., 1995). Similarly, D’Alonzo et al. (1995) showed that capsaicin had anti-arrhythmic and anti-ischaemic activity in perfused isolated hearts from guinea pigs and these effects were attributed to the blocking of voltage-gated Ca\(^{2+}\) channels. It was concluded that capsaicin caused effects similar to those of Ca\(^{2+}\) channel antagonists in perfused hearts (D’Alonzo et al., 1995).

In light of the effects of capsaicin on calcium influx in the rat aortae the effects of oleamide and anandamide on calcium-influx were investigated. This was because the literature shows that endocannabinoids can act directly to inhibit a number of ion channels. Chemin et al. (2001) demonstrated that 1µM anandamide
can block T-type calcium channel current in a receptor independent manner. Anandamide and 2-AG (IC$_{50}$ 2.7µM) have been reported to directly block Shaker-related Kv1.2 potassium channels (Poling et al., 1996). Indeed anandamide, 2-AG, N-palmitoylethanolamine and N-oleoylethanolamine directly blocked human cardiac kv1.5 channels (Barana et al., 2010). Zhang et al., (2000) described a similar effect of 10µM arachidonic acid on T-type Ca$^{2+}$ channels. Arachidonic acid can also inhibit the contractility of ventricular myocytes by inhibiting L-type Ca$^{2+}$ channels in a concentration-dependent manner (IC$_{50}$ 8.5µM) (Liu et al., 2007). In feline cerebral arterial smooth muscle anandamide inhibited L-type Ca$^{2+}$ channels (Gebremedhin et al., 1999). The authors proposed that inhibition of L-type Ca$^{2+}$ channels occurred via CB$_1$ receptors as the inhibitory effect of anandamide was sensitive to both pertusis toxin and rimonabant, while correlating with smooth muscle expression. Similar effects of anandamide via CB$_1$ receptors on Ca$^{2+}$ channels were present in the rat myocardium (Li et al., 2009). Although the literature documents that endocannabinoids can both directly and indirectly block ion channel function, the present chapter demonstrated that the presence of oleamide and anandamide had no effect on calcium influx in rat aortae.

A number of recent studies have described non-TRPV1 mediated capsaicin-induced relaxations. Capsaicin appears to be able to induce relaxations by a variety of mechanisms that appear to differ between tissues. Several studies have implicated the activation of different K$^+$ channels in relaxant responses to capsaicin (Zhu et al., 1997; Yeon et al., 2001; Fujimoto et al., 2006). Sim et al. (2001) demonstrated that capsaicin concentration-dependently relaxed antral circular myocytes from the guinea-pig stomach. It was also shown that capsaicin produced similar inhibitory effects to calcium channel antagonists and caused relaxations by blocking voltage
operated calcium channels (Sim et al., 2001). It was concluded that capsaicin was acting on intracellular stores of calcium. Relaxant responses to capsaicin have also shown to be mediated by NO in human sigmoid colon circular muscle (Bartho et al., 2002) and by COX-2 in human nasal vasculature (Van Crombruggen et al., 2011).

The present chapter, in addition to other non-sensory nerve mediated mechanisms detailed in the literature, has outlined a possible mechanism of action underlying the vascular responses to capsaicin. This helps supports the findings of Chapters 2 and 3 where capsaicin exposure was shown to inhibit the vasorelaxation of aortic segments from Wistar and SHR rats to oleamide and anandamide in a manner distinct from sensory-nerves. It is possible that aortic segments lack TRPV1 expression and that capsaicin exposure causes changes to ion channels that prevent anandamide and oleamide elicited vasorelaxation. It is also possible that capsaicin persists even after the 20 minute wash-out periods and so directly affects ion channels in aortic segments. The possibility that capsaicin may be interfering with a novel CB cannabinoid receptor, which is distinct from the classical cannabinoid receptors, in order to inhibit aortic relaxation to oleamide and anandamide should not be ignored. Indeed, Hoi and Hiley (2006) proposed that in small mesenteric rat arteries oleamide and anandamide may be acting at novel cannabinoid receptor that is sensitive to rimonabant and O-1918 and coupled to a G\textsubscript{i/o} protein.

In summary, the present chapter has demonstrated that capsaicin causes concentration-dependent vasorelaxation of both the rat isolated aorta and the porcine coronary artery. The vascular actions of capsaicin in the rat aorta were mediated by mechanisms distinct from TRPV1 receptors. It has also been shown that capsaicin can block the contractile responses produced by calcium re-introduction in both the rat aorta and porcine coronary arteries. In addition,
capsaicin blocked the contractile effects of Bay-K 8644, an L-type Ca\(^{2+}\) channel activator, in porcine coronary arteries. Thus, we have provided evidence that suggests capsaicin can inhibit calcium-uptake by blocking L-type Ca\(^{2+}\) channels in arteries. In conclusion, we have suggested a mechanism of action for capsaicin driving its vascular responses that is distinct from TRPV1 receptor activation. These results also have implications for experimental pharmacology, namely that treatment with capsaicin should be interpreted with care and that relaxant responses to capsaicin do not necessarily imply a role for sensory nerves.
Chapter 7

Investigating the effects of oleamide in the perfused mesenteric arterial bed
Chapter 7 Investigating the effects of oleamide in the perfused mesenteric arterial bed

7.1 Introduction

As discussed in Chapter 1, the endothelium releases vasoactive mediators that are important in the control of vascular tone. Nitric oxide and prostacyclin are well characterised endothelium-derived vasodilators and account for endothelium-dependent relaxations to agonists such as acetylcholine and bradykinin and to physical stimuli like shear stress. It has been uncovered that an additional endothelium-derived relaxant exists independently of NO and prostacyclin. This additional relaxant is characterised by endothelium-dependent hyperpolarisation of underlying vascular smooth muscle and its effects are abolished by Ca\(^{2+}\)-activated K\(^+\)-channels and hyperpolarising concentrations of extracellular K\(^+\) and is termed endothelium-derived hyperpolarising factor (EDHF) (Taylor and Weston, 1988).

The relative importance of EDHF versus NO appears to be dependent upon vessel size. EDHF-mediated mechanisms appear more important to endothelium-dependent relaxation as vessel size gets smaller, with NO contribution being more important in conduit vessels (Shimokawa et al., 1996; Tomioka et al., 1999). Shimokawa et al. (1996) demonstrated endothelium-dependent vasorelaxation to acetylcholine in the rat isolated aorta and in the proximal and distal mesenteric arteries. The importance of NO to vasorelaxation decreased as vessel size decreased and correlated with lower basal release of NO in resistance vessels and decreased expression of eNOS. Contribution of EDHF-mediated mechanisms was greatest in the relaxation of distal mesenteric arteries. McCulloch et al. (1997) demonstrated that EDHF-mediated
mechanisms may be upregulated in order to compensate for the loss of NO.

EDHF-mediated relaxations to agonists involve an increase in endothelial calcium, causing the activation of Ca\(^{2+}\)-activated K\(^+\)-channels. The resulting efflux of K\(^+\) hyperpolarises endothelial cells and this hyperpolarisation is transmitted to the vascular smooth muscle by the generation of an EDHF. A number of different mechanisms have been implicated as EDHFs. It has been demonstrated that communication through myoendothelial gap-junctions plays an important role in transmitting this hyperpolarisation to the smooth muscle. A host of studies using gap-junction blockers and connexin-mimetic peptides have implicated gap junctions in mediating EDHF-type endothe-lium-dependent relaxations (Chaytor et al., 1998; 2001; 2003; Taylor et al., 1998; Edwards et al., 1999; Griffith and Taylor, 1999; De Vriese et al., 2002; Griffith et al., 2002).

The connexin-mimetic peptide, Gap27, inhibited EDHF-mediated relaxations to acetylcholine in rabbit mesenteric arteries (Chaytor et al., 1998). EDHF-mediated relaxation of rabbit mesenteric arteries is also sensitive to glycyrrhetinic acid derived gap junction blockers (Taylor et al., 1998; Griffith and Taylor, 1999; Chaytor et al., 2000). A range of other connexin-mimetic peptides, each one inhibiting a specific connexin, confirmed the role of gap junctional communication in EDHF-mediated relaxation of the rat hepatic artery and rabbit iliac artery (Chaytor et al., 2001; Griffith et al., 2002).

The EDHF-mediated vasorelaxant effect of carbachol in the perfused rat arterial mesenteric bed was inhibited by the gap-junction blocker 18-α glycyrrhetinic acid (Harris et al., 2000). Palmitoleic acid, clotrimazole and ouabain also attenuated EDHF-type responses in
the mesenteric arterial bed and were shown to blunt cell-to-cell transfer of Lucifer yellow dye (Harris et al., 2000). In the same tissue preparation, Harris et al. (2002) implicated gap-junctional communication in anandamide-induced vasorelaxation. Previously, vasorelaxation of rabbit mesenteric arteries to anandamide was shown to be partially dependent on functioning gap junctions (Chaytor et al., 1999). Interestingly, an extensive body of research has shown that oleamide can block gap-junctional communication (Guan et al., 1997; Boger et al., 1998; Bannerman et al., 2000; Nagasawa et al., 2006). Oleamide (20µM) completely blocked gap-junctional communication in rat glial cells, similarly anandamide (5µM) acted as a gap junction blocker in striatal astrocytes (Venance et al., 1995; Boger et al., 1998).

The aim of this chapter is to characterise the effects of oleamide on the endothelium-dependent relaxation induced by carbachol in the perfused rat mesenteric arterial bed. Since oleamide has been shown to inhibit gap-junctional communication its effects against EDHF-mediated responses were assessed. In light of the fact that anandamide relaxes the rat mesenteric arterial bed (Harris et al., 2002) and oleamide causes vasorelaxation of isolated mesenteric arteries from the rat (Hoi and Hiley, 2006; Sudhahar et al., 2009) it is clearly important to characterise responses to oleamide in the same vessel bed.
7.2 Methods

7.2.1 Animals

Male Wistar rats (200-400g) were stunned with a blow to the head and killed by exsanguination. The rats were dissected with a mid-line incision to reveal the superior mesenteric artery, which was ligated and cleaned of fatty tissue with blunt dissection. A small incision was made in the artery and a cannula (internal diameter 0.58mm; outer diameter 0.96mm) was inserted and ligated below the insertion point. The intestinal wall was carefully cut away from the mesenteric arterial bed. All rats used were housed at the Biomedical Services Unit, University of Nottingham with a 12 h light/dark cycle and in temperature-controlled conditions.

7.2.2 Perfused rat mesenteric arterial bed

The mesenteric arterial bed was then removed and attached to a perfusion system and suspended in a heated jacketed organ-bath. The isolated arterial bed was perfused at 5ml/min with gassed (5% CO\textsubscript{2}/95% O\textsubscript{2}) Krebs-Henseleit solution (NaCl 118, KCl 4.7, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO 4.2, NaHCO\textsubscript{3} 25, D-glucose 10, CaCl\textsubscript{2} 2 (mM)). Pressure was measured by a pressure transducer coupled to a MacLab 4e system.

The isolated arterial bed was allowed to equilibrate for 30 minutes and the α\textsubscript{1} adrenoceptor, methoxamine (1-10µM), was initially added to the perfusion buffer to increase pressure. Methoxamine was present throughout the experiment. After established tone had stabilised, vasorelaxant responses to oleamide (355pmol-3.52µmol) (Figure 7.1) and carbachol (5.48pmol-54.8nmol) were assessed by constructing dose-response curves by administering doses by bolus injections (30µL-330µL) via an injection port in the perfusion system. In order to examine the role of sensory nerves in vasorelaxant
responses to oleamide, arterial beds were perfused with buffer containing capsaicin (10µM) for 1h followed by a 30min washout period. This chronic exposure to capsaicin was designed to exhaust sensory nerves of vasoactive neuropeptides (Zygmunt et al., 1999). The role of NO in relaxant responses to oleamide and carbachol was characterised by the addition of L-NAME (300µM), an inhibitor of nitric oxide synthase, to the perfusion fluid. In addition, L-NAME was present in all other experiments. The importance of K+ channel-dependent mechanisms of vasorelaxation was assessed by constructing dose-response curves in arterial beds being perfused with a high K+ buffer (NaCl 62.5, KCl 59.4, MgSO4 1.2, KH4PO 1.2, NaHCO3 25, D-glucose 10, CaCl2 2 (mM)) (Wheal et al., 2010). Experiments were also performed in the presence of carbenoxolone (100µM), to assess the importance of gap junctional communication in mediating vasorelaxation to carbachol and oleamide (Davidson et al., 1986). In addition, the effect of oleamide (20µM) on the vasorelaxant responses to carbachol was investigated. Dose-response curves to sodium nitroprusside (SNP) (336fmol-1.01nmol), an NO donor, were constructed in the presence of oleamide (20µM), to assess if oleamide affected NO-mediated relaxation or relaxation per se.
Figure 7.1 Representative trace of the relaxant response to doses of oleamide. A=107nmol, B=355nmol, C=1.07µmol and D=3.52µmol.

7.2.3 Drugs

Carbenoxolone (3β-Hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate), carbachol (2-Hydroxyethyl) tri-methylammonium chloride carbamate Carbachol Carbamylcholine chloride), capsaicin (8-methyl-N-vanillyl-trans-6-nonamide), L-NAME (NG-Nitro-L-arginine methyl ester hydrochloride), methoxamine (α-(1-Aminoethyl)-2, 5-dimethoxybenzyl alcohol hydrochloride) and SNP (Nitroprusside sodium SNP Sodium nitroferricyanide Sodium pentacyanonitrosylferrat) were purchased from Sigma Chemical Co. (UK). Oleamide (cis-9 10 octadecenoamide) was supplied by Tocris Co. (UK). Carbenoxolone, carbachol, L-NAME and methoxamine were all dissolved in distilled water. Capsaicin was dissolved in ethanol and oleamide was dissolved in DMSO (Dimethyl sulfoxide).

7.2.4 Statistical analysis

All responses are expressed as mean data with the associated standard error of the mean (S.E.M). The GraphPad Prism 5.0
software (San Diego, CA) was used to plot mean data as sigmoidal concentration-response curves using a sigmoidal equation \[ Y = Bottom + \frac{(Top - Bottom)}{(1 + 10^{((\log EC_{50} - X) \times Hillslope)})}, \] when X=logarithm of agonist concentration and Y=response from Bottom to Top in a sigmoidal shape. The curves were used to determine potency (pED$_{50}$ which is the negative log of the dose causing 50% of the maximal relaxation) and maximal response ($R_{max}$) values. Statistical significance was determined using one-way ANOVA when comparing multiple data-sets. One-way ANOVA was followed by a Bonferroni post hoc carried out as appropriate. P-values <0.05 were considered significant.
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7.3 Results

7.3.1 Vasorelaxant effects of oleamide in the perfused rat mesenteric arterial bed

Oleamide caused dose-dependent vasorelaxation of perfused mesenteric arterial beds isolated from Wistar rats ($R_{\text{max}}=57.6\pm3.9\%$, $pED_{50}=6.73\pm0.08$, $n=3-5$) (Figure 7.2). The vasorelaxant effect of oleamide was unaffected by L-NAME ($R_{\text{max}}=78.3\pm16.3\%$, $pED_{50}=6.40\pm0.24$, $n=4$) (Figure 7.2).

Figure 7.2

![Graph showing vasorelaxation to oleamide in the rat mesenteric arterial bed contracted with methoxamine in the presence of L-NAME and in the combined presence of L-NAME and carbenoxolone. Mean data with bars indicating S.E.M are displayed.](image-url)

Figure 7.2 Vasorelaxation to oleamide in the rat mesenteric arterial bed contracted with methoxamine in the presence of L-NAME and in the combined presence of L-NAME and carbenoxolone. Mean data with bars indicating S.E.M are displayed.
7.3.2 Involvement of gap-junctions in the vasorelaxant response to oleamide in the perfused rat mesenteric arterial bed

The vasorelaxant responses to oleamide (R$_{\text{max}}$=57.6±3.9%, pED$_{50}$=6.73±0.08, n=3) were also unaffected in the presence of both L-NAME and carbenoxolone (R$_{\text{max}}$=65.9±6.6%, pED$_{50}$=7.23±0.17, n=4) (Figure 7.2).

7.3.3 The involvement of potassium channels in the vasorelaxant response to oleamide in the perfused rat mesenteric arterial bed

The contraction of mesenteric arterial beds with high extracellular potassium in the presence of L-NAME abolished vasorelaxant responses to oleamide at doses below (1.07µmole) (Figure 7.3). Whereas, the relaxant response in methoxamine pre-contracted arterial beds was 57.0±11.2% (n=5) in the presence of L-NAME (Figure 7.3). Only at the highest dose of oleamide was there a relaxant response of 30.7±23.1% (n=4) in the presence of high extracellular potassium.
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Figure 7.3

Figure 7.3 Vasorelaxation to oleamide in the rat mesenteric arterial bed after a 1h pre-treatment with capsaicin and after contraction with high extracellular potassium in the presence of L-NAME. Mean data with bars indicating S.E.M are displayed.

7.3.4 Involvement of sensory nerves in the vasorelaxant responses to oleamide in the perfused rat mesenteric arterial bed

The vasorelaxant effects of oleamide were unaffected by 1h capsaicin (10µM) pre-treatment (R\text{max}=58.2±3.9\%,\ pED_{50}=6.62±0.08, n=4) (Figure 7.3).

7.3.5 Endothelium-dependent vasorelaxant of the perfused rat mesenteric arterial bed

Carbachol caused a dose-dependent vasorelaxation of perfused mesenteric arterial beds (R\text{max}=85.4±34.4\%,\ pED_{50}=9.69±1.00, n=4) (Figure 7.4). The presence of 300µM L-NAME caused a
significant (P<0.05) decrease in the vasorelaxant response to carbachol, such that at 54.75nmol, vasorelaxation was decreased from 81.5±5.9% (n=4) to 44.7±7.2% (n=4) (Figure 7.4). The presence of both 20µM oleamide and 300µM L-NAME further decreased (P<0.05) the vasorelaxant response to carbachol, such that at 54.75nmol, vasorelaxation was 10.6±7.2% (n=5) (Figure 7.4). Similarly, the presence of both 100µM carbenoxolone and 300µM L-NAME abolished the vasorelaxant response to carbachol (Figure 7.4).

Figure 7.4

![Graph showing endothelium-dependent vasorelaxation to carbachol in the rat mesenteric arterial bed contracted with methoxamine in the presence of L-NAME, both L-NAME and carbenoxolone, and both L-NAME and oleamide. Mean data with bars indicating S.E.M are displayed.](image_url)
7.3.6 Endothelium-independent vasorelaxation of the perfused rat mesenteric arterial bed

Vasorelaxation caused by SNP in the presence of L-NAME was unaffected by the presence of oleamide. Vasorelaxation to the maximum concentration of SNP used was 85.5±9.2% (n=3), compared to 65.5±5.2% (n=3) in the presence of oleamide (20µM) (Figure 7.5).

Figure 7.5

Figure 7.5 Endothelium-independent vasorelaxation to SNP in the rat mesenteric arterial bed contracted with methoxamine in the presence of L-NAME and oleamide. Mean data with bars indicating S.E.M are displayed. Experiments were analysed using Student’s t-test.
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7.4. Discussion

One of the principal findings of the present chapter is that the endocannabinoid-like substance, oleamide, induces vasorelaxation of the rat mesenteric arterial bed. This supports previous reports showing oleamide to be a potent vasodilator of small resistance mesenteric arteries (Hoi and Hiley, 2006; Sudhahar et al., 2009).

The current set of experiments also demonstrated that oleamide significantly attenuated the endothelium-dependent relaxation of the rat mesenteric arterial bed. The present study shows that oleamide had comparable effects to carbenoxolone at inhibiting carbachol-induced relaxation of the mesenteric arterial bed. Carbenoxolone is a glycyrrhetinic acid derivative that blocked gap-junctional communication in human fibroblast mutant cells in a manner that was not connexion specific (Davidson et al., 1986; Davidson and Baumgarten, 1988). Communication through gap junctions has been reported in a number of EDHF-mediated responses (Yamamoto et al., 1998; 1999; Harris et al., 2000; Coleman et al., 2001). Previously, Harris et al. (2000) described carbachol-induced EDHF-mediated relaxation of the rat isolated mesenteric arterial bed. The EDHF-mediated responses were inhibited by the gap-junction blockers 18 α-glycyrrhetinic acid, palmitoleic acid and clotrimazole (Harris et al., 2000). The previous work by Harris et al. (2000) implicated gap junctional communication in EDHF-type relaxations. This was confirmed in this chapter but, more importantly, it was also found that oleamide specifically interfered with EDHF-type responses whilst leaving nitric oxide-mediated responses unaffected.

Oleamide has been shown to elicit inhibitory effects on gap-junction communication in a number of tissues; including, rat glial cells (Guan et al., 1997; Boger et al., 1998), neural crest cells (Huang et al., 1998; Bannerman et al., 2000), tracheal epithelial cells (Boitano
and Evans, 2000), somniferous tubule cells (Decrouy et al., 2004), endothelial cells of the blood brain barrier (Nagasawa et al., 2006), osteoblasts (Schiller et al., 2001) and osteocytes (Ishihara et al., 2012). Guan et al. (1997) reported that oleamide (50µM) completely blocked gap junction communication in rat glial cells, demonstrated through the inhibition of Lucifer yellow dye transfer and electrical conductance. Oleamide blocked gap junctions in a non-specific manner, inhibiting both α₁ and β₁ connexin subtypes (Guan et al., 1997). Boger et al. (1998) reported a similar effect of oleamide on gap junctions in the same cell type. In addition, it was demonstrated that oleamide (50µM) inhibited neural crest cell migration in mice embryonic cells, a process regulated by gap-junction communication (Huang et al., 1998). Interestingly, oleamide (25-100µM) inhibited the function of endothelial cells from the blood brain barrier, mimicking the effects of the well known gap junction blocker 18β-glycyrrhetinic acid (Nagasawa et al., 2006).

The vasorelaxant effect of oleamide in the rat mesenteric arterial bed was reduced after contraction with high extracellular K⁺ (60mM) instead of methoxamine. This suggests a role for K⁺-channels in oleamide-induced vasorelaxation, possibly through EDHF-mediated mechanisms. However, the responses to oleamide were unaffected by carbenoxolone, which, as discussed above, interferes with EDHF-type responses. Therefore, these findings suggest that, in the mesenteric arterial bed, oleamide does not act via EDHF release and its effects against carbachol-induced relaxation would suggest that it interferes with EDHF. So the present findings do not identify a mechanism of relaxation to oleamide but it would appear to be independent of nitric oxide and sensory nerves. The findings with high extracellular potassium may implicate a K⁺ channel-dependent mechanism and further experiments using selective K⁺ channel inhibitors are required to establish this.
Harris et al. (2002) characterised the vasorelaxation of the rat mesenteric arterial bed to the prototypical endocannabinoid, anandamide. Anandamide, similarly to oleamide in the present study, caused vasorelaxation independently of NO production (Harris et al., 2002). In contrast, Hoi and Hiley, (2006) had reported a NO-dependent component of oleamide-induced vasorelaxation. Anandamide caused relaxation that was sensitive to the presence of 18α-glychrrhetinic acid, possibly implicating gap junctions in the response (Harris et al., 2002). Anandamide has previously been demonstrated to interact with gap junctions in striatal astrocytes (Venance et al., 1995). However, anandamide-induced vasorelaxation was insensitive to both carbenoxolone and palmitoleic acid (Harris et al., 2002). The authors postulated that responses to anandamide may have involved the sodium pump and not gap junctions as ouabain also had an inhibitory effect.

Endocannabinoids can also act as TRPV1 agonists, activating the release of neuropeptides, such as CGRP, from perivascular sensory nerves (Zygmont et al., 1999). Sensory-nerve mediated activity has been implicated in oleamide-induced relaxation of isolated mesenteric arteries (Hoi and Hiley, 2006; Sudhahar et al., 2009). Sudhahar et al. (2009) demonstrated that the presence of capsazepine, a TRPV1 antagonist, reduced the potency of oleamide and that mesenteric arteries were associated with the expression of TRPV1. Vasorelaxant responses to anandamide in the rat mesenteric arterial bed were attenuated by capsaicin pre-treatment and in the presence of ruthenium red (Harris et al., 2002). It was therefore hypothesised that oleamide was causing vasorelaxation through sensory-nerve mediated activity. However, this proposal can be rejected as a chronic pre-treatment of the mesenteric arterial bed with capsaicin had no effect on oleamide-induced responses.
This chapter presents evidence demonstrating oleamide to be a potent vasorelaxant of the rat mesenteric arterial bed. However, its key vascular effect could be via its ability to impair endothelium-dependent relaxations, possibly via inhibition of gap junctions. This could be of significance as endogenous oleamide might regulate endothelial function and this could be relevance to vascular pathology. For example under pathological conditions when levels of oleamide are increased or following inhibition of FAAH there may be inhibition of EDHF-type responses.
Chapter 8

General Discussion
Chapter 8 General Discussion

8.1 General Discussion

This study aimed to characterise the vascular actions of the endocannabinoid-like mediator oleamide, making a comparison with the actions of the prototypical endocannabinoid anandamide. This thesis investigated the effects of oleamide in large conduit vessels in both health and disease, providing insights into the mechanisms of action. This study also characterised the properties of oleamide in a whole arterial bed and its effects on endothelium-dependent relaxation.

8.1.1 Oleamide as a vasorelaxant

The vascular actions of oleamide have received relatively little interest, especially in comparison to the cardiovascular effects of anandamide. One of the major aims of this thesis was to build on previous research demonstrating the potent vasorelaxant properties of oleamide in small resistance mesenteric arteries (Hoi and Hiley, 2006; Sudhahar et al., 2009). Chapter 2 aimed to characterise and compare the vascular actions of oleamide and anandamide in larger conduit vessels.

An initial finding of this study was the small vasorelaxant effect of oleamide in the rat isolated aorta. Oleamide induced approximately 20% relaxation, which is comparable to the effect of other cannabinoid compounds in the same preparation (O’Sullivan et al., 2005). In the present study anandamide failed to elicit any response in aortae, contradictory to the small vasorelaxation reported elsewhere. The vasorelaxant effect of oleamide was found to be tissue dependent, as oleamide did not induce responses in porcine isolated coronary and mesenteric arteries. Anandamide, in contrast, evoked relaxation of mesenteric arteries in a NO-
dependent manner. Therefore, this study demonstrates clear differences in the mechanisms of action between oleamide and anandamide.

Oleamide-induced vasorelaxation was abolished by capsaicin pre-treatment independently of sensory-nerve mediated activity. Results in Chapter 5 demonstrate that capsaicin can affect vascular control by mechanisms distinct from sensory-nerve activation. Hence, the results suggest that oleamide may have an additional unidentified capsaicin-sensitive site of action. This action may also play an important role in the enhanced effects of oleamide and anandamide in aorta from hypertension.

Building on previous work by others using the rat isolated mesenteric artery, this thesis demonstrated that oleamide caused a large vasorelaxant effect in the rat perfused mesenteric arterial bed. This response was diminished by a depolarising concentration of high extracellular $K^+$, possibly implicating the involvement of $K^+$-channels and/or EDHF-mediated activity. This provides supporting evidence for previous reports demonstrating the sensitivity of the oleamide response in rat mesenteric arteries to a combination of apamin and charybdotoxin and also to high extracellular $K^+$ (Hoi and Hiley, 2006).

8.1.2 Alternative mechanism of action for capsaicin

As discussed in Chapter 6, capsaicin has been shown to be a potent agonist of TRPV1 receptors, through which it can exert a range of pharmacological effects. The activation of TRPV1 on perivascular sensory nerves by capsaicin can result in the release of vasoactive neuropeptides causing vasorelaxation. Thus, chronic exposure of isolated vessels to capsaicin is a routinely used pharmacological tool to exhaust neuropeptide stores and desensitise sensory nerves to TRPV1 activation. For example, Zygmunt et al. (1999) pre-treated
isolated arteries with capsaicin to help implicate the involvement of sensory-nerves in anandamide-induced vasodilation. Therefore, this study similarly exposed arteries to capsaicin in order to characterise the involvement of sensory nerves in responses to oleamide and anandamide. This thesis demonstrates the capsaicin-sensitive nature of both oleamide-induced vasorelaxation of isolated rat aortae and in the enhanced effects of oleamide in aortae from hypertension. Similarly, the augmented effect of anandamide in aortae from SHR rats was normalised by capsaicin pre-treatment. However, it was demonstrated that the vascular effects of oleamide and anandamide were insensitive to TRPV1 antagonism with capsazepine and ruthenium red. This suggested that capsaicin, and therefore oleamide and anandamide, were eliciting vascular effects independently of TRPV1 and sensory nerves. This prompted the investigation into the mechanisms of action of capsaicin in the rat aorta and in the porcine coronary artery.

Other studies demonstrated that capsaicin can induce changes in vascular tone by mechanisms distinct from TRPV1 receptors, by acting directly on ion channels (Lo et al., 1995; Ellis et al., 1997; Zhu et al., 1997; Yeon et al., 2001; Fujimoto et al., 2006). This thesis contributes further evidence demonstrating that capsaicin evokes vasorelaxation via mechanisms independent of sensory nerves. It was shown that capsaicin-induced vasorelaxation of both rat aortae and porcine coronary arteries was insensitive to the presence of capsazepine and ruthenium red. In both porcine coronary arteries and rat aortae the presence of capsaicin inhibited the contractile response caused by the re-introduction of calcium. It was also demonstrated that the vasoconstrictor properties of Bay-k 8644, a L-type calcium channel activator, in porcine coronary arteries was blocked by capsaicin. Thus this thesis presents an alternative mechanism of action for capsaicin in affecting arterial tone and substantiates previous work in which capsaicin inhibited
calcium-influx in vascular smooth muscle from rat aortae (Monsereenusorn and Kongsamut, 1985; Lo et al., 1995).

The demonstration of an alternative mechanism of action for capsaicin helps validate results in Chapter 2 and 3 in which capsaicin pre-treatment attenuated vasorelaxation by anandamide and oleamide. How capsaicin and its newly defined ability to either directly or indirectly block L-type calcium channels can interfere with endocannabinoid-induced effects is not completely understood. There are a number of possible explanations however. Firstly, capsaicin may persist after the washout period and act directly at ion channels to interfere with endocannabinoid-induced responses. Secondly, capsaicin may interact with a novel, as yet unidentified, CB or TRPV receptor to attenuate relaxation evoked by oleamide and anandamide. Another alternative is that capsaicin is interfering with an ion channel that is a downstream target of a novel CB receptor. Importantly, this thesis provides results which have some implications for experimental pharmacology. Most notably that capsaicin may stimulate effects in tissue lacking TRPV1 receptors and sensory-nerve innervation and so experiments utilising chronic capsaicin pre-treatment should validate results using TRPV1 antagonists.

8.1.3 Effect of hypertension on oleamide-induced responses

A major focus of this thesis was to examine the effects of hypertension on oleamide-induced vasorelaxation. Having demonstrated a small vasorelaxant response to oleamide in normal Wistar aortae in Chapter 2 and in light of the enhanced cardiovascular effects of anandamide in hypertension reported elsewhere, the present study aimed to characterise the effects of oleamide in aortae from the SHR model of hypertension. The principal finding of Chapter 3 was that oleamide-induced vasorelaxation was greatly augmented in aortae from hypertension
compared to those from normotensive controls. This augmented response in hypertension is related to differences in the COX-pathway. This conclusion was based upon the observation that the presence of indomethacin potentiated the relaxant effects of oleamide in aortae from the normotensive strain to a level comparable to arteries from hypertension. This thesis also describes upregulated responses to anandamide in SHR aortae, consistent with responses reported by Wheal and Randall (2009). However, alterations in prostanoid metabolism were not responsible for the elevated vasorelaxation caused by anandamide in hypertension. The augmented responses to oleamide and anandamide in aortae from hypertension were both normalised to levels comparable to those from normotensive controls by chronic exposure to capsaicin. Similarly to the capsaicin-sensitive nature of oleamide-induced relaxation in Wistar aortae, enhanced responses to oleamide and anandamide are independent of TRPV1 activity.

Another interesting point of discussion is the effect of indomethacin on oleamide-induced relaxations in different tissues. In WKY aorta indomethacin enhanced responses to oleamide. Therefore, in the WKY control an alteration to the COX-pathway exists that is not present in the SHR. Similarly, this effect of indomethacin on oleamide responses was not present in the normal Wistar aorta, suggesting that the Wistar and SHR may be more similar than the SHR and WKY control. This opens up the debate whether the alteration in the COX pathway is lost in SHR rats as an adaptive change to hypertension or if it is due to the WKY being an inappropriate control animal. Indeed, whether or not the WKY is an appropriate control has been discussed by others in greater detail. It has been demonstrated that SHR and WKY rats are genetically quite different and in genes unrelated to hypertension, which is probably due to random genetic drift (St. Lezin et al., 1992). In the
future it would be useful to investigate the effects of oleamide using transgenic models of hypertension.

Having described enhanced responses in aortae from hypertension, the focus of Chapter 5 was to examine the vascular effects of anandamide and oleamide in a different arterial preparation. In contrast to augmented responses in aortic rings from SHR rats, oleamide caused similar vasorelaxation in mesenteric arteries from both healthy and diseased rats. Thus, this thesis demonstrates the tissue specific nature of the effects of hypertension on oleamide-induced responses. The enhanced vasorelaxant effect of anandamide in hypertension was however also observed in mesenteric arteries.

This study also documents differences in endothelial function between vessels from hypertensive and normotensive rats, although underlying differences in endothelial function did not account for the enhanced effects of oleamide and anandamide in SHR aortae. Chapter 4 demonstrates greater endothelium-dependent relaxations in aortae from SHR rats. This thesis therefore presents results that are contradictory to earlier work suggesting that hypertension is associated with impaired endothelial function (Luscher and Vanhoutte, 1986). A substantial amount of research showed diminished responses to endothelium-dependent relaxants and the production of prostanoid-derived EDCF in SHR aortae. As discussed in Chapter 4, the contradictory nature of these results may reflect age-related changes in endothelial function. It is possible that enhanced endothelial function could represent an adaptive change in the early stages of established hypertension. In both SHR and WKY aortae carbachol-induced relaxations were inhibited by L-NAME, however a small residual component of endothelium-dependent relaxation in SHR aortae was insensitive to L-NAME. This residual component of endothelium-dependent
relaxation was also independent of sensory nerves, EDHF, COX-pathway, gap-junctions and hydrogen peroxide. Chapter 5 provides further evidence for augmented endothelial function in the early stages of established hypertension in the SHR, carbachol was identified to be a more potent vasorelaxant of SHR mesenteric arteries versus those from WKY rats.

8.1.4 Effect of oleamide on EDHF-mediated vasorelaxation

Gap-junctional communication was originally implicated in EDHF-mediated endothelium-dependent vasorelaxation by Chaytor et al. (1998). The role of gap junctions in EDHF-type responses is discussed in detail in Chapter 7. Indeed, the endothelium-dependent relaxation induced by carbachol in the perfused rat mesenteric arterial bed was sensitive to gap-junction inhibition by 18-α glycyrrhetinic acid, palmitoleic acid, clotrimazole, ouabain and rimonabant (Harris et al., 2000). This thesis provides results that confirm the findings of Harris et al. (2000) and the role of gap-junctions in EDHF-mediated relaxation. The presence of carbenoxolone, a glycyrrhetinic acid derived gap-junction blocker, in combination with L-NAME completely blocked the relaxant actions of carbachol in the perfused rat mesenteric arterial bed. This study also provided evidence substantiating the ability of oleamide to act as a gap-junction blocker. Guan et al. (1997) originally described oleamide’s ability to inhibit gap-junctional communication in rat glial cells, subsequent groups used oleamide as a tool to study the role of gap-junctions in physiological processes. The present study suggests that oleamide can act at gap junctions expressed in the vasculature and function to block EDHF-type relaxations. Oleamide replicated the effects of carbenoxolone in the presence of L-NAME, in blocking vasorelaxant response to carbachol in the perfused mesenteric arterial bed. Further studies should include the effects of oleamide on Lucifer dye transfer in vascular tissue. Future
investigations elucidating the specific-gap junctions involved in EDHF-mediated relaxation of rat mesenteric arterial bed and the connexins expressed in this tissue should help identify the specific gap junction complexes at which oleamide acts.

8.1.5 Conclusions

This thesis contributes to existing literature demonstrating the vasorelaxant properties of oleamide. The investigation shows that oleamide exerts a small vasorelaxant effect in the rat isolated aorta, which is not as large or as potent an effect reported elsewhere in rat mesenteric resistance arteries (Hoi and Hiley, 2006; Sudhahar et al., 2009). However, in porcine coronary and mesenteric arteries oleamide did not demonstrate vasorelaxant properties. This clearly shows a tissue and species dependent nature of the vasodilator effects of oleamide. Results presented here suggest that oleamide is less effective in conduit vessels compared to resistance vessels. Oleamide elicited a large vasorelaxant response in the rat perfused mesenteric arterial bed, building on work by other research groups describing relaxation of isolated mesenteric arteries to oleamide.

A major finding of this study was the greatly enhanced response to both anandamide and oleamide in aortae from hypertensive rats compared to those from normotensive controls. The COX-pathway was shown to be an important component in regulating oleamide-induced responses in aortae for normotensive WKY rats. The limiting effect of the COX-pathway on oleamide responses was absent in hypertension and it is possible that this represents an adaptive change in the early stages of established hypertension. This thesis also highlights the tissue specific nature of the effect of hypertension on oleamide-induced vasorelaxation, with oleamide-induced relaxation of mesenteric arteries being equal between the two strains of rat.
Chapter 8 General Discussion

The augmented responses to both oleamide and anandamide were sensitive to capsaicin pre-treatment but were shown to be independent of sensory-nerve mediated activity. Vasorelaxation of normal Wistar aortae was also abolished by capsaicin. This thesis provides a capsaicin-sensitive mechanism, independent from sensory-nerves and neuropeptide release, which can mediate vascular responses to endocannabinoids in conduit arteries. The nature of this novel mechanism of action remains to be elucidated.

In summary, oleamide has been found to exert significant vascular effects, both directly and indirectly by interfering with EDHF. The enhanced responses to oleamide and endothelium-dependent relaxations found in early hypertension may represent an important adaptive mechanism to modulate cardiovascular control in hypertension.
Figure 8.1 Potential vascular mechanisms of action of oleamide. Oleamide may act to inhibit EDHF-mediated endothelium-dependent vasorelaxation by blocking myoendothelial gap junctions. Oleamide may also be able to cause vasorelaxation via K⁺ channels, which may also involve EDHF-mediated mechanisms. The cyclooxygenase (COX) pathway can play an important role in regulating the vasorelaxant effect of oleamide. Oleamide and anandamide induce capsaicin-sensitive vasorelaxation, which may point to the existence of a capsaicin-sensitive novel CB-receptor, most likely located on the vascular smooth muscle. It has been demonstrated that
capsaicin can block Ca\(^{2+}\)-uptake through L-type calcium channels on vascular smooth muscle.
References


Oxide is involved in the relaxant effect of capsaicin in the human sigmoid colon circular muscle. Naunyn Schmiedebergs Arch Pharmacol 366, 496-500.


contributions of NO and gap junctional communication to endothelium-dependent relaxations of rabbit resistance arteries vary with vessel size. Microvasc Res 63, 115-128.


Coleman, H.A., Tare, M., Parkington, H.C., 2001. EDHF is not K+ but may be due to spread of current from the endothelium in guinea pig arterioles. Am J Physiol Heart Circ Physiol 280, H2478-2483.


De Angelis, A., Rinaldi, B., Capuano, A., Rossi, F., Filippelli, A., 2004. Indomethacin potentiates acetylcholine-induced vasodilation by increasing free


References


Ford, R.J., Rush, J.W., Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO and EDCF dependent. Am J Physiol Heart Circ Physiol 300, H64-75.


Fulton, D., Quilley, J., 1998. Evidence against anandamide as the
hyperpolarizing factor mediating the nitric oxide-independent coronary vasodilator effect of bradykinin in the rat. J Pharmacol Exp Ther 286, 1146-1151.


Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. J Neurosci 17, 5327-5333.


Harris, D., Martin, P.E., Evans, W.H., Kendall, D.A., Griffith, T.M., Randall, M.D., 2000. Role of gap junctions in endothelium-derived...


Herrera-Solis, A., Vasquez, K.G., Prospero-Garcia, O., Acute and subchronic administration of anandamide or oleamide increases REM sleep in rats. Pharmacol Biochem Behav 95, 106-112.


Hillard, C.J., Edgemond, W.S., Campbell, W.B., 1995. Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a
References


References


Jackson, W.F., Konig, A., Dambacher, T., Busse, R., 1993. Prostacyclin-


Liu, S.J., 2007. Inhibition of L-type Ca2+ channel current and negative inotropy induced by arachidonic acid in adult rat ventricular myocytes. Am J Physiol Cell Physiol 293, C1594-1604.


Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron 31, 463-475.


References


Miura, H., Gutterman, D.D., 1998. Human coronary arteriolar dilation to arachidonic acid depends on cytochrome P-450 monooxygenase and Ca2+--


References

Mol Aspects Med 26, 33-65.


O'Sullivan, S.E., Kendall, D.A., Randall, M.D., 2005. Vascular effects of
References

delta 9-tetrahydrocannabinol (THC), anandamide and N-arachidonoyldopamine (NADA) in the rat isolated aorta. Eur J Pharmacol 507, 211-221.


through endothelial TRPV1 receptor activation in the rat arterial mesenteric bed. J Physiol 568, 539-551.


Biophys Res Commun 229, 114-120.


Rapoport, R.M., Williams, S.P., 1996. Role of prostaglandins in acetylcholine-induced contraction of aorta from spontaneously hypertensive and Wistar-Kyoto rats. Hypertension 28, 64-75.


Ros, J., Claria, J., To-Figueras, J., Planaguma, A., Cejudo-Martin, P., Fernandez-Varo, G., Martin-Ruiz, R., Arroyo, V., Rivera, F., Rodes, J., Jimenez,
References


Sim, J.H., Kim, Y.C., Kim, S.J., Lee, S.J., Suh, S.H., Jun, J.Y., So, I.,


Diabetes 51, 1157-1165.


Suzuki, S., Takeshita, A., Imaizumi, T., Hirooka, Y., Yoshida, M., Ando,


References


Ueda, N., Kurahashi, Y., Yamamoto, S., Tokunaga, T., 1995. Partial purification and characterization of the porcine brain enzyme hydrolyzing and
References


Virdis, A., Colucci, R., Versari, D., Ghisu, N., Fornai, M., Antonioli, L.


References

endotoxic shock. Anal Biochem 294, 73-82.


Yang, D., Gluais, P., Zhang, J.N., Vanhoutte, P.M., Feletou, M., 2004. Endothelium-dependent contractions to acetylcholine, ATP and the calcium ionophore A 23187 in aortas from spontaneously hypertensive and...


9. Appendices

9.1 Appendix 1

9.1.1 Pre-contraction data for Chapter 2

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Appendices

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Values are mean±S.E.M, with n values stated in brackets.

9.1.2 Pre-contraction data for Chapter 3

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<td>SHR-oleamide (n=6)</td>
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<td>WKY-anandamide (n=10)</td>
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<td>Control</td>
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<tr>
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<td>L-NAME</td>
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*Group abbreviations:* WKY = Wistar-Kyoto rat, SHR = spontaneously hypertensive rat.
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<th>SHR</th>
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## Appendices

<table>
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<th>Basal tone (g)</th>
<th>Induced tone (g)</th>
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<td>0.72±0.13</td>
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Values are mean±S.E.M, with n values stated in brackets.

### 9.1.3 Pre-contraction data for Chapter 4

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<td>SHR (n=4)</td>
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### Appendices

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<th>Basal tone (g)</th>
<th>Induced tone (g)</th>
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<td>+ L-NAME + high K⁺ (n=3)</td>
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<td>capsaicin pre-treat + L-NAME + high K⁺ (n=3)</td>
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Values are mean±S.E.M, with n values stated in brackets.

#### 9.1.4 Pre-contraction data for Chapter 5

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Values are mean±S.E.M, with \( n \) values stated in brackets.

9.1.5 Pre-contraction data for Chapter 6

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Values are mean±S.E.M, with \( n \) values stated in brackets.

9.1.6 Pre-contraction data for Chapter 7

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<th>Figure</th>
<th>Treatment</th>
<th>Basal tone (mmHg)</th>
<th>Induced tone (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>control (n=3-5)</td>
<td>32.89±2.49</td>
<td>61.75±11.86</td>
</tr>
<tr>
<td>7.2</td>
<td>+ L-NAME (n=4)</td>
<td>34.94±1.75</td>
<td>99.72±14.36</td>
</tr>
<tr>
<td>7.2</td>
<td>+ carbenoxolone + L-NAME</td>
<td>44.19±5.00</td>
<td>37.44±6.30</td>
</tr>
<tr>
<td>7.3</td>
<td>+ capsaicin pre-treatment</td>
<td>31.67±2.79</td>
<td>73.00±8.16</td>
</tr>
<tr>
<td>7.3</td>
<td>+ L-NAME + High K(^+) (n=4)</td>
<td>54.49±16.26</td>
<td>73.66±21.37</td>
</tr>
<tr>
<td>7.4</td>
<td>control (n=4)</td>
<td>32.81±3.22</td>
<td>77.36±17.25</td>
</tr>
<tr>
<td>7.4</td>
<td>+ L-NAME (n=4)</td>
<td>34.94±1.75</td>
<td>95.24±5.41</td>
</tr>
</tbody>
</table>
### Appendices

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Value</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>+ oleamide + L-NAME (n=5)</td>
<td>44.67±2.79</td>
<td>99.17±16.32</td>
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<tr>
<td>7.4</td>
<td>+ carbenoxolone + L-NAME (n=5)</td>
<td>44.19±5.00</td>
<td>40.66±7.65</td>
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<tr>
<td>7.5</td>
<td>control (n=3)</td>
<td>41.40±4.06</td>
<td>56.21±8.19</td>
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<tr>
<td>7.5</td>
<td>+ oleamide (n=3)</td>
<td>42.34±3.54</td>
<td>58.30±11.33</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M, with n values stated in brackets.