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Cannabinoid Signaling in Health and Disease

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Abstract

Cannabis sativa has long been used for medicinal purposes. To improve safety and efficacy, compounds from *C. sativa* were purified or synthesized and named under an umbrella group as cannabinoids. Currently, several cannabinoids may be prescribed in Canada for a variety of indications such as nausea and pain. More recently, an increasing number of reports suggest other salutary effects associated with endogenous cannabinoid signaling including cardioprotection. The therapeutic potential of cannabinoids is therefore extended; however, evidence is limited and mechanisms remain unclear. In addition, the use of cannabinoids clinically has been hindered due to pronounced psychoactive side effects.

This review provides an overview on the endocannabinoid system, including known physiological roles, and conditions in which cannabinoid receptor signaling has been implicated.

Keywords: endocannabinoid, cannabinoid receptor, anandamide, 2-arachidonoylglycerol, FAAH, MAGL

1. Introduction

Cannabis sativa has long been used to relieve symptoms such as pain, fever, anxiety, and diarrhea in the context of numerous diseases (Grant et al. 2012). To improve safety and efficacy, compounds from *C. sativa* were purified or synthesized and named under an umbrella group as cannabinoids. Currently, several cannabinoids may be prescribed in Canada to mitigate nausea from chemotherapy, relieve pain from cancer, prevent spasticity due to multiple sclerosis and improve appetite in patients with AIDS (Kalant et al. 2012). More recently, an increasing number of studies investigated the endogenous cannabinoid system and discovered other possible uses, including cardioprotection (Pacher et al. 2006). The therapeutic potential of cannabinoids is therefore extended; however, evidence is limited and mechanisms remain unclear. In addition, the use of cannabinoids clinically has been hindered due to pronounced psychoactive side effects, such as dizziness, euphoria and addiction (Grant et al. 2012). Clearly, the role of cannabinoids and the endocannabinoid system in cardiac function and diseases remains poorly understood. Furthermore, any attempts to develop cannabinoid-based cardiovascular therapies would require mitigation of adverse psychoactive effects. This review covers a variety of (patho)physiological effects of the endocannabinoid system, with emphasis on cannabinoid receptor subtype-specific roles in multiple disease states.

2. Endocannabinoid system.

The endocannabinoid system is a lipid signaling system that is involved in a wide range of physiological and pathological processes such as energy metabolism and inflammation. Three major components constitute the endocannabinoid system: endocannabinoids, cannabinoid receptors, and endocannabinoid metabolism (Battista et al. 2006). Endocannabinoids are endogenously-produced bioactive lipids that activate cannabinoid receptors. N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG), which were initially identified in brain and intestine respectively (Devane et al. 1992; Mechoulam et al. 1995), are the best-studied naturally-occurring endocannabinoids.

Anandamide was first discovered in porcine brain (Devane et al. 1992), and was then detected in other tissues from a variety of species: bovine and rat brain, rat and human spleen, rat skin and testis of rat, as well as in the human heart (the latter, at approximately 10 pmol/g) (Felder et al. 1996). Endogenous anandamide synthesis is a two-step process (Figure 1): i) N-arachidonoyl phosphatidylethanolamine (NAPE) formation by transfer of arachidonic acid from the SN-1 position of phosphatidylcholine to the ethanolamine moiety of phosphatidylethanolamine (Sugiura et al. 1996), and ii) anandamide generation from the cleavage of NAPE by NAPE phospholipase D (Basavarajappa 2007). In fact, NAPE distribution in various regions of rat brain corresponds with that of anandamide, although NAPE concentrations are much higher (Bisogno et al. 1999). Formation of NAPE is catalyzed by N-acyltransferase (NAT). In rat cortical neurons, NAT activity is regulated by Ca^{2+} and cAMP (Cadas et al. 1996). An increase in intracellular Ca^{2+} level stimulates activity of NAT, and this activity is potentiated by a cAMP activator, such as foskolin, with subsequent cAMP-dependent protein kinase (PKA) stimulation (Cadas et al. 1996). The key role of Ca^{2+} in NAT activation

was also confirmed by Cadas et al. In addition, they observed other modulators of NAT such as stimulation by dithiothreitol, and inhibition by phenylmethane sulfonyl fluoride and dithionitrobenzoic acid (Cadas et al. 1997). The second step (i.e. cleavage of NAPE) is carried out by phospholipase D, which is activated by interaction between dopamine D_2 receptors and the Rho family of small G proteins (Senogles 2000). Anandamide synthesis and release are rapid processes; for instance, anandamide synthesis and release from rat hippocampus neurons occur within 75-190 ms and in fewer than 50 ms upon stimulation at 22 °C and 37 °C, respectively (Heinbockel et al. 2005).

2-AG was isolated from canine intestine (Mechoulam et al. 1995) and was later detected by Kondo et al. in rat brain (3.36 nmol/g), liver (1.15 nmol/g), spleen (1.17 nmol/g), kidney (0.98 nmol/g) and lung (0.78 nmol/g). Note that the brain contains higher concentrations (Kondo et al. 1998). Stella et al. also determined the concentration of 2-AG in rat brain (4 nmol/g), and this is approximately 170 times greater than that of anandamide (23 pmol/g) (Stella et al. 1997). In fact, the amount of 2-AG is higher than anandamide in most tissues (Mechoulam et al. 1998). There are also two steps in 2-AG synthesis (Figure 1): i) diacylglycerol (DAG) is generated by phospholipid C-regulated hydrolysis of membrane phospholipids, followed by ii) DAG lipase-catalyzed conversion to 2-AG (Basavarajappa 2007). 2-AG synthesis is stimulated in response to increased intracellular Ca^{2+} concentration. For example, ionomycin, a Ca^{2+} ionophore, significantly increased 2-AG concentration in hippocampus slices (Stella et al. 1997). Additionally, others reported that 2-AG synthesis might be initiated by receptor agonism. Stella et al. found 2-AG synthesis was stimulated by Ca^{2+} entry through glutamate N-methyl-D-aspartate (NMDA) receptor channels (Stella and Piomelli 2001), and NMDA receptor activation was reported to enhance activities of phospholipase C and DAG lipase (Stella and Piomelli

2001). Moreover, acetylcholine receptor activation, which activates phospholipase C, increased 2-AG production in rat aorta (Mechoulam et al. 1998).

It is believed that anandamide and 2-AG are synthesized on demand upon stimulation (for example, by depolarization of postsynaptic neurons and resultant intracellular Ca^{2+} accumulation (Stella et al. 1997)), and released immediately due to their lipophilicity (Battista et al. 2012; Hashimotodani et al. 2013).

2.1. Cannabinoid receptors

2.1.1. CB1/CB2 receptors

Two GPCRs for endocannabinoids, CB1 and CB2, have been extensively studied to date. CB1 receptors are highly expressed in brain (Matsuda et al. 1990), except in the respiratory centers of the brain stem (Howlett et al. 2002). They are particularly dense in cerebral cortex, hippocampus, basal ganglia and cerebellum. CB1 receptors are also expressed in peripheral sensory nerves and the autonomic nervous system (Quarta et al. 2010). In addition, CB1 receptors are present at measurable levels in several peripheral tissues, including the spleen, lung, thymus, heart (Bonz et al. 2003; Howlett et al. 2002) and vasculature (Gebremedhin et al. 1999; Liu et al. 2000).

In contrast, CB2 receptors are abundantly expressed in hematopoietic cells (Valk and Delwel 1998) and in the immune system (Munro et al. 1993), including spleen, tonsils, bone marrow, and leukocytes. Bouchard et al. reported that expression levels of CB2 receptors are comparable to that of CB1 receptors in rat hearts (Bouchard et al. 2003). It was originally believed that CB2 receptors are absent from the brain (Munro et al. 1993). However, CB2 receptor mRNA and protein expression were eventually detected in rat and mouse central nervous systems (CNS), including neurons in various regions of the brain (Skaper et al. 1996), although at a much lower level compared to CB1 receptors (Gong et al. 2006; Van Sickle et al.

2005). CB2 receptors have since been detected in cerebrovascular endothelial cells (Golech et al. 2004), microglia (Beltramo et al. 2006) and neurons, with postsynaptic localization (Brusco et al. 2008; Callen et al. 2012; Kim and Li 2015).

Existing evidence suggests that both CB1 and CB2 receptors are coupled to $G\alpha_{i/o}$ protein. Thus, activation of CB1 and CB2 receptors inhibits adenylyl cyclase (AC)/cAMP/PKA/ERK signaling (Demuth and Molleman 2006; Jung et al. 1997). CB1, but not CB2 receptors, also activate $G\alpha_s$ proteins, and stimulate cAMP production (McAllister and Glass 2002). These dual inhibition and activation effects of the CB1 receptor on AC/cAMP were demonstrated to be ligand-specific (Bonhaus et al. 1998). Furthermore, CB1 receptors reportedly modulate Ca^{2+} channels. First, activation of CB1 receptors on presynaptic neurons inhibited Ca^{2+} influx through N-type Ca^{2+} channels (Mackie et al. 1993) and hence suppressed neurotransmitter release (Shen and Thayer 1998). CB1 receptor activation also inhibited Ca^{2+} current through L-type Ca^{2+} channels in arterial smooth muscle cells leading to vasodilation (Gebremedhin et al. 1999). Second, activation of CB1 receptors stimulated Ca^{2+} release from internal stores in astrocytes and endothelial cells (Fimiani et al. 1999), and this was likely regulated by phospholipase C and downstream cascades. In fact, the CB1 receptor may couple with $G_{q/11}$ and trigger the phospholipase C pathway (Lauckner et al. 2005). In human endothelial cells, augmented Ca^{2+} release via CB1 agonism was coupled to increased activity of nitric oxide synthase, and this may explain vasodilation induced by anandamide (Fimiani et al. 1999; Mombouli et al. 1999).

Anandamide exhibits marked selectivity for CB1 over CB2 receptors (Felder et al. 1995; Khanolkar et al. 1996), whereas 2-AG is less selective (Ben-Shabat et al. 1998; Mechoulam et al. 1995). To investigate the specific role of each receptor, analogues of endocannabinoids have been synthesized (Pertwee 2006) including, for example, the CB1-selective agonist arachidonyl-

2-chloroethylamide (ACEA) (Hillard et al. 1999), and the CB2-selective agonist JWH-133 (Marriott and Huffman 2008). Synthetic agonists and antagonists are listed in Tables 1 and 2, respectively.

2.1.2. Other putative cannabinoid receptors

Some anandamide effects cannot be explained by CB1/CB2 activation, suggesting that there might exist other cannabinoid receptors (Brown and Robin Hiley 2009).

G protein-coupled receptor 55 (GPR55) has gained attention as a potential receptor for cannabinoid ligands that mediated effects independently of CB1 and CB2 receptors. In fact, it was recommended as a potential candidate as a third CB receptor (i.e. CB3) (Brown and Robin Hiley 2009). GPR55 was detected in human brain and peripheral tissues including spleen, adrenal gland and intestine (Yang et al. 2016). Although considered a potential cannabinoid receptor, GPR55 exhibits a different ligand profile from classical CB1/CB2 receptors. Ryberg et al. reported that anandamide, 2-AG, Δ^9-tetrahydrocannabinol (THC), HU210 (CB1 agonist), and AM251 (CB1 antagonist) act as agonists of GPR55. Cannabidiol, which has a restricted affinity for CB1 and CB2 receptors, acts as an antagonist, whereas WIN55, 212-2 (CB1/CB2 agonist) and AM281 (CB1 antagonist) exert neither agonistic nor antagonistic effects (Ryberg et al. 2007). In addition, GPR55 elicits signaling cascades distinct from those of CB1/CB2 receptors. GPR55 activation stimulates the $G\alpha_{12/13}$ pathway (Yang et al. 2016), and downstream effectors include RhoA/Rho-associated protein kinase (ROCK) and then c-Jun N-terminal kinases (JNK) and p38 MAPKs (Nishida et al. 2005), as well as PLC-induced Ca^{2+} release and subsequent transcriptional modification via nuclear factor of activated T-cells (NFAT) (Henstridge et al. 2009).

Transient receptor potential vanilloid type 1 (TRPV1) receptors are non-selective cation channels that also mediate some endocannabinoid effects. Found in central and peripheral neurons (Ross 2003; Van Der Stelt and Di Marzo 2004; Zygmunt et al. 1999), as well as non-neuronal cells (Fernandes et al. 2012), they are activated by naturally-occurring vanilloids, acid and heat, and signal a painful and burning sensation. For example, myocardial ischemia causes acidification, which then activates TRPV1 and leads to angina pain (Huang et al. 2009). Anandamide and ACEA also activate TRPV1 (Ross 2003). Toth et al. summarized features of the interaction between TRPV1 and anandamide: the efficacy of anandamide on TRPV1 activation depends on tissue, species, TRPV1 expression level and phosphorylation status; the concentration of anandamide required to activate TRPV1 is higher (≈10 times) than that required to activate CB1; metabolites of anandamide may activate TRPV1; activation of TRPV1 stimulates anandamide synthesis; and CB1-dependent cascades activated by anandamide (e.g. PKA or MAPKs) stimulate TRPV1 activation (Toth et al. 2009). The complex interaction between endocannabinoids and TRPV1 renders mechanisms of endocannabinoid-TRPV1 crosstalk difficult to elucidate.

In summary, CB1 and CB2 receptors are broadly distributed and are involved in a wide range of physiological processes. In addition, cannabinoid compounds also activate GPR55 and TRPV1 receptors, making them putative cannabinoid receptors. The profile of cannabinoid receptors requires further investigation.

2.2. Endocannabinoid transport and degradation

Endocannabinoid activity is rapidly terminated by cellular uptake and intracellular degradation.

The transport mechanism of anandamide and 2-AG is not completely understood, although hypotheses of passive diffusion and protein transporter facilitated diffusion have been proposed (Basavarajappa 2007). Beltramo et al. identified an anandamide membrane transporter in rat neurons and astrocytes, and AM404, a competitive inhibitor of anandamide transport, prolonged and enhanced anandamide-stimulated CB1 activity (Beltramo et al. 1997). Fu et al. also discovered an anandamide-selective transport protein in rat brain and liver. It is an analogue of fatty acid amide hydrolase (FAAH), though it lacks hydrolytic activity, and is therefore named FAAH-like anandamide transporter (FLAT). A competitive FLAT inhibitor, ARN272, also generated CB1-mediated analgesic and anti-inflammation effects by suppressing the cellular uptake of anandamide (Fu et al. 2012).

Two major endocannabinoid-metabolizing enzymes are known: FAAH for anandamide and 2-AG (Deutsch and Chin 1993; Maccarrone et al. 1998), and monoacylglycerol lipase (MAGL) for 2-AG (Dinhet al. 2002; Dinh et al. 2002; Saario et al. 2004). These enzymes hydrolyze anandamide to arachidonic acid and ethanolamine, and 2-AG to arachidonic acid and glycerol, which are recycled to form phospholipids that might integrate into the cell membrane (Figure 1) (Basavarajappa 2007).

FAAH is widely expressed in many tissues, such as brain, liver, lung, spleen, testis, and kidney. Its expression was not detected in skeletal muscle and heart (Cravatt and Lichtman 2002), yet myocardial anandamide levels were elevated in FAAH knockout mice; this provides indirect evidence for the presence of FAAH in the heart (Pacher et al. 2004), perhaps at levels below detection limits. In mouse brain and liver, anandamide hydrolysis rates dropped by 100 and 50 fold respectively in FAAH knockout mice (Cravatt et al. 2001). Regulation of FAAH activity has been considered an important approach to manipulate the endocannabinoid system. To date, little

evidence of a receptor-dependent regulatory mechanism exists, but many efforts have been made toward synthesis of chemical stimulators and inhibitors of FAAH (Faure et al. 2014; Lodola et al. 2015).

MAGL mRNA was also detected in a number of rat tissues, including adipose tissue, kidney, brain, heart, lung, liver, skeletal muscle and spleen (Karlsson et al. 1997). Blankman et al. reported that MAGL contributes to 85% of 2-AG hydrolysis in mouse brain, whereas FAAH only accounts for 1% (Blankman et al. 2007). MAGL is reportedly upregulated by peroxisome proliferator-activated receptor α (PPARα), at least at the transcriptional level in mouse hepatocytes (Rakhshandehroo et al. 2007).

Other enzymes in addition to FAAH and MAGL reportedly degrade anandamide and 2-AG, but their activity is less clear compare to that of MAGL and FAAH (Basavarajappa 2007; Pertwee 2014). These include FAAH-2, N-acylethanolamine-hydrolyzing acid amidase (NAAA), α/β hydrolase domain (ABHD), cyclooxygenase-2 (COX-2), and cytochrome p450. FAAH-2 was identified in primate models, including humans, but not in rodents. However, its hydrolytic activity for anandamide is approximately 38 times lower than FAAH (Wei et al. 2006). NAAA was also identified in various human, rat, and mouse tissues (eg. lung, spleen and small intestine). Using N-palmitoylethanolamine, an anandamide analogue, as reference, rat FAAH catalytic activity shows a preference towards anandamide (V_{max}=5700 nmol/min/mg; K_m=30 μM) over N-palmitoylethanolamine (V_{max}=1800 nmol/min/mg; K_m=70 μM) (Katayama et al. 1999), whereas rat NAAA hydrolase activity for anandamide was only 8% of that for N-palmitoylethanolamine (V_{max}=1847 nmol/min/mg; K_m=35 μM) (Ueda et al. 2001), suggesting that NAAA exerts weak hydrolase activity for anandamide compared to FAAH. Furthermore, NAAA exhibits no hydrolase activity on 2-AG (Ueda et al. 2001). Thus, it is reasonable to conclude that the role of

NAAA as a hydrolase of endocannabinoids is insignificant.

Proteins that contain the ABHD (i.e. ABHD 6 and ABHD 12) are serine hydrolases that can hydrolyze 2-AG. In fact, ABHD 6 and ABHD 12 account for 4% and 9% of total 2-AG hydrolysis in mouse brain, respectively (Blankman et al. 2007). COX-2 is well known for its ability to convert arachidonic acid to the pro-inflammatory lipid, prostaglandin. COX-2 also oxygenates anandamide and 2-AG to produce ethanolamide and glycerol ester derivatives of prostaglandin respectively (Rouzer and Marnett 2011). Likewise, various families of cytochrome p450 (e.g. 3A4, 4F2, and 2D6) oxidize anandamide into different isoforms of epoxyeicosatrienoic acids ethanolamides (EET-EAs) and hydroxy-eicosatetraenoic acids ethanolamides (HETE-EAs), which exhibit diverse effects on inflammation and vascular tone (Rouzer and Marnett 2011). Cytochrome p450 was only recently implicated in 2-AG oxidation in 2014, when McDougle et al. detected 2-AG metabolite production (i.e. 2-EET-glycerols) by CYP2J2, a predominant cytochrome p450 in the heart. CYP2J2 also hydrolyzes 2-AG to glycerol and arachidonic acids in a NADPH-dependent manner (McDougle et al. 2014). Reports of COX-2- and cytochrome p450-dependent metabolism of endocannabinoids have only recently emerged and represent novel research areas that warrant further investigation.

The aforementioned enzymes responsible for degrading endocannabinoids play an important role in terminating endocannabinoid signaling. Indeed, manipulating the levels of FAAH and MAGL by overexpression, knockdown or using their inhibitors has been an area of intense study. For example, Hohmann et al. reported that inhibition of MAGL and FAAH increased 2-AG and anandamide levels in rat brain and enhanced anti-hyperalgesic effects (Hohmann et al. 2005). Ho et al. found that FAAH and MAGL inhibitors enhanced the vasodilatory actions of anandamide and 2-AG in rat isolated small mesenteric arteries (Ho and

Randall 2007). Carnevali et al. demonstrated an anti-depressant effect of FAAH inhibition, which was associated with increases in central and peripheral anandamide levels (Carnevali et al. 2015).

Collectively, endocannabinoid signaling includes endocannabinoid biosynthesis, receptor activation, membrane transport and degradation. Each of these represents a potential therapeutic target by which the endocannabinoid system might be manipulated.

3. (Patho-)physiological functions of the endocannabinoid system

Cannabis sativa, which stimulated interest in the endocannabinoid system, contains more than 60 phytocannabinoids. Among them, THC and cannabidiol are considered the major components (Szaflarski and Bebin 2014). THC mainly activates CB1 receptors, which are abundantly expressed in central nervous system, and accounts for the psychoactive effects of cannabis use (Pertwee 2008). It is now widely accepted that THC activates CB1 receptors located on presynaptic terminals, and this leads to inhibition of neurotransmitter release. Conversely, activated CB1 may stimulate release of neurotransmitters such as dopamine, from other neurons. These dual inhibitory and stimulatory actions of THC on neurotransmitter release might provide an explanation for the complex mood effects (for example, euphoria vs. anxiety) that might be observed after cannabis use (Pertwee 2008). Cannabidiol, in contrast, lacks psychoactivity. It exhibits low affinities for both CB1 and CB2 receptors, and may in fact act instead as an inverse agonist of CB1 receptors (Thomas et al. 2007). Consistent with this notion, cannabidiol prevented THC-induced psychotic responses in humans (Bhattacharyya et al. 2010). Finally, *C. sativa* has also been used to relieve nausea and diarrhea.

Considering the physiological processes influenced by *C. sativa*, as well as the wide distribution of endocannabinoids and their receptors throughout the body, the endocannabinoid

system has been implicated in multiple physiological and pathological processes. Whether acting at CB receptor or non-CB receptor sites, cannabinoid-related compounds exert inhibitory effects on obesity, inflammation, pain, and chemotherapy-induced nausea or vomiting, and may alleviate the symptoms of neurodegenerative diseases and multiple sclerosis. Nabilone, dronabinol, and sativex are cannabinoid-based drugs that have been approved to treat pain, appetite loss, spasticity and chemotherapy-induced nausea (Grant et al. 2012). However, it should be noted that cannabinoids are linked to undesirable side effects, particularly psychoactive in nature. Therefore, the endocannabinoid system is a convergence of benefits and risks that requires careful and comprehensive study.

3.1. Appetite and energy expenditure

Central CB1 receptors play an important role in appetite regulation. Kirkham et al. found that injection of 2-AG to limbic forebrain, a brain area that controls eating motivation, stimulated appetite, and this was inhibited by rimonabant (SR141716), an CB1 antagonist (Kirkham et al. 2002). Appetite stimulation by CB1 receptors was confirmed by Cota et al., who observed reductions in food intake and body weight in CB1-deficient mice (Cota et al. 2003). In contrast, FAAH-deficient mice exhibited enhanced appetite and this was accompanied by elevated anandamide levels in hypothalamus, liver, and small intestine (Tourino et al. 2010). The stimulatory effect on appetite corresponds with the finding that anandamide and 2-AG levels in limbic forebrain and hypothalamus were highest during fasting, but dropped during eating (Kirkham et al. 2002). A clinical trial on dronabinol, a cannabinoid-based drug, showed improved appetite in AIDS patients, and dronabinol was subsequently approved to treat AIDS-associated anorexia (Beal et al. 1995).

In addition to appetite regulation, CB1 receptors also modulate energy expenditure. Verty

et al. reported that CB1 blockade with rimonabant in rats enhanced thermogenesis in brown adipose tissue, and this was associated with up-regulation of UCP1, a protein that stimulates heat production. These changes were partially attenuated by denervation, implying the role of central CB1 receptors in restricting energy expenditure (Verty et al. 2009). A clinical trial that involved obese patients with hypertension or dyslipidemia showed that rimonabant (20 mg/day) reduced body weight and waist circumference, and also improved several cardiovascular metabolic parameters (i.e. increased high-density lipoprotein and decreased triglycerides and insulin resistance) (Van Gaal et al. 2005). These findings were in agreement with similar clinical trials on rimonabant (Pi-Sunyer et al. 2006; Scheen et al. 2006). In 2006, rimonabant was released into the European market as an anti-obesity drug, although it was quickly withdrawn due to reports of adverse effects such as nausea, depression and anxiety (Di Marzo and Despres 2009).

To eliminate the psychiatric effects mediated by central CB1 receptors, attempts were made to evaluate the contribution of peripheral CB1 receptors to obesity. Cluny et al. observed a transient reduction in food intake and sustained body weight loss in response to a peripherally-restricted CB1 receptor antagonist, AM6545, in rats and mice (Cluny et al. 2010). Reduced hepatic triglycerides, increased expression of fatty acid oxidation genes, and improved insulin sensitivity were also confirmed with AM6545 (Tam et al. 2010). Hsiao et al. compared the effects of rimonabant and BPR0912, a peripherally-restricted CB1 receptor antagonist, on obesity parameters in diet-induced obese mice. Similar reductions in body weight, serum insulin, triglycerides, and hepatic triglycerides were observed with both compounds, but compared to rimonabant, BPR0912 raised fatty acid oxidation-related gene expression and thermogenesis more significantly (Hsiao et al. 2015). In summary, activation of CB1 receptors contributes to increased appetite, suppressed energy expenditure, and when deranged, obesity. Therefore,

antagonism of CB1 receptors, particularly in the periphery, remains a promising approach to enhance adipocyte lipolysis, reduce food intake and decrease body weight (Arrabal et al. 2015; Mastinu et al. 2012).

The role of CB2 receptors in food intake and energy metabolism is not as extensively studied as that of CB1, but appears to oppose CB1 effects. The observation that the inhibitory effect of AM6545 on food intake was abolished in CB1/CB2 knockout mice, but not in CB1 knockout mice indicated that CB2 receptors might be involved (Cluny et al. 2010). Onaivi et al. reported increased appetite in C57Bl/6 mice treated with the CB2 receptor antagonist AM630 after 12 h food-deprivation, but not in other strains (i.e. Balb/c and DBA/2) (Onaivi et al. 2008). Similarly, the CB2 receptor agonist JWH015 induced a transient reduction in food intake in C57Bl/6 mice, which was restored by AM630. JWH-015 also induced body weight loss, reduced white adipose tissue weight and adipocyte cell size, and increased triglyceride lipase expression (Verty et al. 2015).

3.2. Inflammation

As CB2 receptors are abundantly expressed in the immune system (Munro et al. 1993), the involvement of CB2 receptors in inflammation is well-documented. Indeed, CB2 receptors play an important role in preventing gastrointestinal inflammation. Storr et al. reported that the CB2 receptor agonists, JWH133 and AM1241, attenuated colitis in mice, and pretreatment with a CB2 receptor antagonist or CB2 knockout abrogated this effect (M. Storr et al. 2009). Similar findings were observed in human colonic mucosa, where tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)-induced inflammation, as evidenced by luminal epithelial and crypt damage and increased lymphocyte density, was attenuated by CB2 activation (Harvey et al. 2013). Overexpression of CB2 receptors in intestine was detected in models of inflammatory

bowel disease, suggesting the important role of CB2 receptors as a compensatory anti-inflammatory response (M. Storr et al. 2009; Wright et al. 2005).

Anti-inflammatory actions of CB2 receptors were also observed in other tissue or cells. For example, anandamide and 2-AG alleviated inflammation in cultured human retinal explant, which was reflected by increased viability of retinal neurons and Muller glia, as well as reduced Muller glia proliferation. This anti-inflammatory effect was achieved by inhibiting proinflammatory cytokines (e.g. IL-6, interferon-γ (IFN-γ), and TNF-α) while elevating anti-inflammatory molecules (e.g. IL-10 and transforming growth factor-β (TGF-β)) (Krishnan and Chatterjee 2012). CB2 receptor activation also relieved rheumatoid arthritis symptoms via multiple pathways, including inhibition of fibroblast proliferation, suppression of proinflammatory cytokine release in fibroblast-like synoviocytes, T cells and macrophages, as well as prevention of bone erosion via stimulating osteoblasts and reducing osteoclasts (Gui et al. 2015).

Incidentally, the anti-inflammatory properties of cannabinoids are also an important aspect of treating neurodegenerative diseases, and cardiovascular risk factors, where inflammation is a key factor. This is discussed below.

3.3. Cancer

CB1 and CB2 receptors have been detected in a variety of cancer cell lines, including astrocytoma (Sanchez et al. 2001), breast cancer (Caffarel et al. 2010), prostate cancer (Chung et al. 2009), lung cancer (Preet et al. 2008) and so on. Greater content of CB1 or CB2 is also associated with increased tumor malignancy (Chung et al. 2009; Gustafsson et al. 2011; Orellana-Serradell et al. 2015; Sanchez et al. 2001).

Cannabinoids may prevent or ameliorate cancer development first by inhibiting tumor growth. Numerous studies observed apoptosis-inducing effects of cannabinoids and their synthetic analogues in a variety of cancer types. For example, THC and WIN-55, 212-2 inhibited the progression of malignant gliomas in rats and mice by inducing apoptotic signaling; possible mechanisms include cannabinoid receptor-activated ceramide accumulation and Raf1/ERK1/2 activation (Galve-Roperh et al. 2000). Selective activation of CB2 receptors also triggered apoptosis within glioma by increasing ceramide synthesis (Sanchez et al. 2001). Moreover, combining a cannabinoid, either THC or cannabidiol, with temozolomide (TMZ), a conventional glioma therapy, significantly enhanced antitumor action in a mouse glioma model, as evidenced by reduced tumor volume compared to TMZ alone (Torres et al. 2011). The role of CB1 receptors in stimulating apoptosis was validated in breast cancer and prostate cancer cultures (primary and commercial cell lines) (De Petrocellis et al. 1998; Orellana-Serradell et al. 2015). Molecular signaling involves ERK activation and Akt inhibition (Orellana-Serradell et al. 2015). Akt inhibition pathway was confirmed further in an *in vivo* study, in which THC and JWH-133, by activating CB2 receptors, suppressed breast tumor growth and lung metastases in a mouse model of malignant breast cancer (Caffarel et al. 2010). Furthermore, *in vitro* and *in vivo* studies reported that both CB1 and CB2 are involved in anti-tumor actions in colon cancer, and TNF-α contributes to CB1/CB2-mediated apoptosis by increasing ceramide production and caspase 3 apoptotic signaling (Cianchi et al. 2008).

Metastasis may also be mitigated by cannabinoids. Met-F-AEA, a metabolically stable analogue of anandamide, suppressed the adhesion and migration of a human breast cancer cell line, and these actions were attributable to CB1-mediated inhibition of focal adhesion kinase (FAK) (Grimaldi et al. 2006). Suppression of the RhoA-ROCK pathway also contributes to the

ability of CB1 to inhibit human breast and prostate cancer cell invasion and migration (Laezza et al. 2008; Nithipatikom et al. 2012). Similar findings were reported in lung cancer cell lines, where THC significantly reduced cell migration, and this was accompanied with inhibition of epidermal growth factor (EGF), FAK, ERK1/2, JNK1/2, and AKT (Preet et al. 2008). Finally, Hinz et al. reported that activation of CB1 and CB2 enhanced expression of tissue inhibitors of matrix metalloproteinases (TIMP-1) in cervical cancer cells which would reduce extracellular matrix degradation, and thereby attenuate metastasis.

3.4. Emesis

Emesis can be triggered centrally or peripherally. Stimuli such as food toxins and chemotherapeutic agents evoke vomiting primarily by inducing serotonin (5-HT) release from the epithelium of the gastrointestinal tract. By activating 5-HT receptors in afferent nerves, signals are communicated to the emesis center in the medulla, followed by a series of motor responses. This emesis center can also be directly activated by central stimuli, for instance, aversive memories (Becker 2010). Cannabinoids have traditionally been used to treat nausea and vomiting. Nabilone and dronabinol, synthetic analogues of THC, are approved to treat chemotherapy-induced vomiting (Sharkey et al. 2014). A clinical trial compared dronabinol, ondansetron (5-HT antagonist), and their combination on chemotherapy-induced nausea and emesis, and found similar anti-emetic effects, However, dronabinol alone showed a better effect on reducing the severity of nausea (Meiri et al. 2007). Sativex, a 1:1 combination of THC and cannabidiol, is not yet approved to treat chemotherapy-induced vomiting. However, a clinical trial observed that in combination with standard anti-emetic therapy, sativex improved chemotherapy-induced nausea and vomiting compared to standard therapy alone (Duran et al. 2010). The role of the endocannabinoid system in anti-emetic effects was investigated in animal

models. Hu et al. showed that CB1 receptor activation inhibited enterotoxin-induced 5-HT release from the intestine of musk shrew, suggesting a peripheral action of CB1 in the anti-emetic effect of cannabinoids (Hu et al. 2007). The role of CB1 receptors was confirmed by other studies (Darmani 2001; O'Brien et al. 2013), but evidence on CB2 receptors is lacking.

3.5. Pain

Pain is regulated by the endocannabinoid system at both central and peripheral sites. Following electrical stimulation, Walker et al. detected release of anandamide in periaqueductal gray, the primary brain region for pain modulation, which coincided with the central CB1-mediated analgesic effect (Walker et al. 1999). Clapper et al. reported that URB937, a peripheral FAAH inhibitor, generated a peripheral accumulation of anandamide, and suppressed both neuropathic and inflammatory pain-related behavior responses as well as neuron activation in the spinal cord; a CB1 receptor antagonist reversed these effects. The ability of URB937 to modulate pain signals despite its lack of CNS penetration implies that activation of peripheral CB1 receptors exhibits an analgesic effect by blocking the transduction of pain signals into the CNS (Clapper et al. 2010). The CB2 receptor was originally found to suppress pain sensation by attenuating the release of pro-inflammatory molecules which increase the sensitivity of primary afferent neurons (Malan et al. 2003). Beltramo et al. later observed a direct inhibition of pain neurotransmitter production by CB2 receptors as well, in parallel to its analgesic effects in a rat model of neuropathic pain and a mouse model of central sensitization (Beltramo et al. 2006). Finally, Romero et al. confirmed the analgesic actions of CB1 and CB2 receptors, in a manner that requires activation of peripheral adrenergic receptors by norepinephrine (Romero et al. 2013).

3.6. Endocannabinoid signaling in the CNS

The endocannabinoid system has been extensively studied in the brain. Anandamide is found in brains of animal models at concentrations of 173 pmol/g, 101 pmol/g and 30 pmol/g in porcine, bovine and rat brain, respectively (Bisogno et al. 1999; Schmid et al. 1995). Felder et al. measured anandamide concentrations in specific regions of human brain, and identified a range of approximately 35 pmol/g in cerebellum to 107 pmol/g in hippocampus (Felder et al. 1996). A brief overview on the important role of endocannabinoid signaling in a few CNS disorders is discussed below.

3.6.1. Multiple sclerosis

Multiple sclerosis is an autoimmune disorder characterized by axon demyelination of neurons within CNS. Cannabis has traditionally been used to relieve symptoms, and current thinking attributes the beneficial effects to immunosuppressive and neuroprotective properties of cannabinoid receptors. However, whether cannabinoid ligands delay multiple sclerosis progression remains unknown, though preclinical studies are supportive. For example, microglial cells, which are the primary immune cells in the CNS, can be activated to secrete pro-inflammatory factors, including IL-12 and IL-23, thereby contributing to the progression of multiple sclerosis. Anandamide inhibited secretion of IL-12 and IL-23 in microglial cells, at least in part through a CB2-dependent ERK1/2 and JNK pathway (Correa et al. 2009). An increase in anandamide concentration was detected in inflammatory brain tissue from multiple sclerosis patients (Eljaschewitsch et al. 2006). Furthermore, in experimental autoimmune encephalomyelitis mice, a model of multiple sclerosis, WIN55, 212-2 (CB1/CB2 agonist) attenuated the up-regulation of inflammatory cytokines (COX-2, iNOS and TNF-α) and microglial-induced cell aggregation in spinal cord and brainstem. These effects of WIN55, 212-2

were reversed by a CB1 receptor antagonist (de Lago et al. 2012). Increasing 2-AG levels in mice spinal cord using a MAGL inhibitor also slowed the progression of multiple sclerosis, and was associated with decreased leukocyte infiltration and microglial activity (Hernandez-Torres et al. 2014). However, the Cannabinoid Use in Progressive Inflammatory brain Disease (CUPID) trial failed to show benefits of dronabinol on multiple sclerosis progression, perhaps due to slow progression rate which confounded statistical detection of group differences (Zajicek et al. 2013).

3.6.2. Neurodegenerative diseases

Parkinson's disease, Huntington's disease, and Alzheimer's disease are three common neurodegenerative diseases characterized by progressive degeneration and/or death of neurons. No therapy has been discovered to cure these diseases yet. However, manipulation of the endocannabinoid system has shown promising effects towards alleviating symptoms.

3.6.2.1. Parkinson's disease

Price et al. showed that administration of WIN55, 212-2 improved survival of dopamine-producing neurons in a mouse model of Parkinson's disease (i.e. 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced), in parallel with improved motor performance. This was mediated by CB2 receptors, whose expression was increased in the disease model (Price et al. 2009). Similar findings were observed in a lipopolysaccharide-induced mouse model of Parkinson's disease (Garcia et al. 2011). Unlike the beneficial effects of CB2 receptor activation, CB1 contributed to disease progression. Rimonabant, a CB1 antagonist, improved motor coordination, but did not alter neurodegeneration (Gonzalez et al. 2006; Kelsey et al. 2009). In addition, neuroprotection provided by some cannabinoid agents such as THC, cannabidiol, and AM404, is due to antioxidant actions (Garcia-Arencibia et al. 2007). For example, cannabidiol, a naturally-occurring cannabinoid with limited affinity for CB1 and CB2 receptors, attenuated dopamine

reduction and increased SOD expression (Garcia-Arencibia et al. 2007). In addition, two anandamide uptake inhibitors, AM404 and UCM707, which would increase anandamide levels, were compared. AM404, which possesses antioxidant properties, rescued dopamine levels in a rat model of Parkinson's disease, whereas UCM707, which lacks antioxidant properties, did not (Garcia-Arencibia et al. 2007). In summary, agonism of CB2 receptors, antagonism of CB1 receptors, and cannabinoids that exert antioxidant activity might improve symptoms of Parkinson's disease.

3.6.2.2. Huntington's disease

Huntington's disease is a genetic neurodegenerative disease that is characterized by impaired muscle coordination and cognitive ability, as well as behavior changes, such as anxiety, depression, apathy and aggression. It is caused by a mutation of the Huntington gene, which leads to neuron degeneration mainly in the striatum. No treatment is known to slow disease progression. Cannabinoid compounds may exert symptom-relieving effects. The malonate-induced rat model of Huntington's disease is associated with increased proinflammatory molecules, edema, and microglial activity. A combination of THC and cannabidiol reversed all of these in a CB1- and CB2-dependent manner (Valdeolivas et al. 2012). The role of CB2 receptors was confirmed in CB2 knockout mice, which responded more severely to malonate (Sagredo et al. 2009). CB1 receptor activity declines dramatically in basal ganglia and striatum during the progression of Huntington's disease, and this decline was proposed as a contributor to disease progression. However, markedly reduced CB1 levels render the CB1 receptor a poor therapeutic target (Lastres-Becker et al. 2001), although cannabinoid treatments (anandamide, methanandamide, and ACEA) increase CB1 mRNA in mouse striatal progenitor cell lines that model features of Huntington's disease (Laprairie et al. 2013). A double-blind, placebo-

controlled, cross-over clinical trial involving 44 patients with Huntington's disease showed that while nabilone failed to improve motor score, the chorea score was improved significantly (Curtis et al. 2009).

3.6.2.3. Alzheimer's disease

Alzheimer's disease is characterized by the excessive deposition of β-amyloid peptide and activation of microglial cells in senile plaques, which lead to neuron degeneration mainly in the hippocampus and prefrontal cortex. Symptoms include cognitive impairment, memory loss, mood swings, behavior changes and so on. Both CB1 and CB2 receptors were detected in senile plaques (Ramirez et al. 2005). CB2 receptor agonism attenuated β-amyloid-induced microglia activation and microglia-induced neurotoxicity in rats, and it preserved cognitive ability (Ramirez et al. 2005). However, studies on CB1 receptors in the progression of Alzheimer's disease are controversial. Some found CB1 is detrimental to memory and learning ability, fostering interest in CB1 antagonism as a treatment approach. For example, Mazzola et al. reported that the CB1 antagonist, rimonabant, reversed β-amyloid peptide-induced memory deficit in mice (Mazzola et al. 2003). In contrast, others demonstrated beneficial effects of CB1 receptors. First, CB1 levels are markedly reduced in brains of various animal models with Alzheimer's disease (Aso et al. 2012; Ramirez et al. 2005). Also, in patients, CB1 activity is increased in the earlier stage of Alzheimer's disease, followed by a reduction in advanced stages of the disease. This implies an initial compensatory response mediated by CB1, which was impaired as neurodegeneration developed (Manuel et al. 2014). Second, Aso et al. showed that the CB1 agonist ACEA, at a non-amnesic dose, prevented cognitive retardation in a mouse model of Alzheimer's disease, particularly in the early stage. Mechanisms include inhibition of glycogen synthase kinase 3β (GSK-3β), microglial activation, and subsequent release of pro-

inflammatory factors (Aso et al. 2012). There is little evidence derived from clinical trials to support the use of cannabinoid-based compounds to treat Alzheimer's disease. However, dronabinol improved adverse psychiatric effects such as agitation, insomnia, and appetite loss in a few small clinical trials (Ahmed et al. 2015).

3.6.3. Mood

It is well known that marijuana use elicits a feeling described as "high." In fact, this is a complex of psychoactive effects due mainly to THC and cannabidiol, the major cannabinoids in marijuana (Fitzgerald et al. 2013). Thus, it was speculated that the endocannabinoid system mediates anti-depressant and anxiolytic effects. The role of CB1 receptors is well-established. The anti-depressant properties of low dose WIN55, 212-2 in rat were blocked by a CB1 receptor antagonist (Bambico et al. 2007). Injection of anandamide and a CB1 selective agonist, ACEA, into midbrain dorsolateral periaqueductal gray, a region that regulates anxiety responses, also elicited anxiolytic effects in rats, whereas a CB1 antagonist abolished these effects (Moreira et al. 2007). However, in these two studies, high doses of cannabinoids failed to elicit the same effects. In fact, evidence suggests that the effects of cannabinoids on anxiety are bidirectional; anxiolytic at low doses whereas anxiogenic at high doses (Rubino et al. 2008; Viveros et al. 2005). In addition, Rubino et al. found that the anxiety-regulation profile of cannabinoids varies in different brain regions. For example, low and high doses of THC injected into the prefrontal cortex and ventral hippocampus elicit anxiolytic and anxiogenic effects, respectively; however, low doses of THC in basolateral amygdala generate anxiogenic effects whereas high doses of THC were ineffective (Rubino et al. 2008).

Despite the complex activity profile of cannabinoids on anxiety and depression, it is consensus thinking that disruption of CB1 signaling leads to depressive- and anxiogenic-like

responses (Moreira et al. 2009). Also, activation of CB1 receptors contributes to the removal of aversive memories (Marsicano et al. 2002). Therefore, CB1 inhibition causes retention of aversive memories and may exacerbate depressive feelings. Patients treated with rimonabant, a CB1 antagonist, as an anti-obesity drug, exhibited depression and anxiety symptoms, and even increased risk of suicide, leading to withdrawal from the market (Christensen et al. 2007).

In summary, both suppression and hyperactivity of the endocannabinoid system may elicit adverse effects, such as anxiety and depression. These effects are mediated by CB1 receptors in the CNS, which should be considered during drug development.

3.6.4. Sleep

Cannabis use has long been associated with improved sleep, and a variety of cannabinoids, either naturally-occurring or synthetic compounds, promote sleep. For example, elevation of anandamide levels, achieved by blocking hydrolysis or direct injection, prolonged sleep duration by increasing non-rapid eye movement (NREM) sleep and rapid eye movement (REM) cycles, and this was interrupted by a CB1 antagonist (Mendez-Diaz et al. 2013; Murillo-Rodriguez et al. 2008). Similarly, administration of CP47, a CB1 agonist, or inhibition of endocannabinoid degradation, stabilized NREM sleep as evidenced by increased NREM bout duration. In contrast, the CB1 antagonist AM281 fragmented NREM without reducing overall sleep time. Rimonabant, also a CB1 antagonist, disturbed REM sleep in rats (Santucci et al. 1996), and CB1 knockout mice exhibited reduced non-rapid eye movement (NREM) sleep (Pava et al. 2014). In fact, rimonabant was withdrawn as an anti-obesity drug due to severe psychoactive side effects, among which insomnia is very common (Nathan et al. 2011). Mechanisms underlying CB1-dependent sleep promotion is largely unknown, though elevated adenosine (Murillo-Rodriguez et al. 2003) and c-Fos expression (Murillo-Rodriguez et al. 2008) might be involved. Collectively,

these findings demonstrated the role of CB1 in maintenance of NREM sleep stability (Pava et al. 2016).

Clinical studies were conducted to evaluate the effects of medical cannabinoids on sleep quality of patients with neuropathic pain, multiple sclerosis, and cancer. While there is reportedly risk of bias in some studies, including non-validated measurements of sleep and failure to blind participants to their treatments, there are reports of positive outcomes such as improved sleep quality, fewer nightmares, reduced sleep interference, etc. (Gates et al. 2014).

3.7. Endocannabinoid system and the cardiovascular system

Components of the endocannabinoid system are elevated in various aspects of cardiovascular disease, including atherosclerosis, myocardial infarction and cardiac hypertrophy (Duerr et al. 2013; Lin et al. 2015). The following sections discuss the potential roles of endocannabinoids and their receptors in regulation of cardiovascular health.

3.7.1. Hemodynamic parameters

Marijuana use leads to blood pressure changes, and the influence of endocannabinoids on hemodynamics has been extensively studied. However, the results are complex. THC induced biphasic changes in blood pressure and heart rate in anesthetized rats, which were characterized by an immediate and transient blood pressure increase followed by a marked drop and prolonged hypotension and bradycardia (Lake et al. 1997). Intravenous injection of anandamide caused a three-phase hemodynamic change in anesthetized rats, including i) phase 1 – a transient reduction in blood pressure, heart rate and cardiac contractility, ii) phase 2 - an elevation of diastolic blood pressure and blood flow in mesenteric and renal vascular beds, followed by iii) phase 3 - a more prolonged and significant decrease in blood pressure and contractility, and a slight reduction in heart rate (Malinowska et al. 2001; Pacher et al. 2004). Other synthetic

compounds, such as HU210, WIN55, 212-2, and CP-55940, also induced prolonged hypotension and bradycardia, although without the initial phases that were observed with THC and anandamide (Lake et al. 1997).

Possible mechanisms include CB1 or CB2 receptor activation, TRPV1 activation and metabolite-induced pathways. A similar three-phase action was observed with methanandamide, a stable analogue of anandamide, indicating the involvement of cannabinoid receptors. In addition, the CB1 receptor antagonist, rimonabant, blocked the phase 3 response, but not the transient pressor effect of THC nor the first two phases of anandamide. This suggests that CB1 is responsible for the prolonged hypotension and bradycardia (Lake et al. 1997; Malinowska et al. 2001), perhaps by suppressing the sympathetic nervous system (Niederhoffer et al. 2001). In contrast, a TRPV1-selective antagonist diminished the phase 1 responses induced by anandamide and methanandamide, suggesting that TRPV1 mediates the initial transient drop of blood pressure and heart rate (Malinowska et al. 2001). Phase 2 may also be induced by TRPV1 receptors, as evidenced by the observation that capsaicin, a potent TRPV1 agonist, also generated the phase 2 increase in blood pressure in anesthetized rats, and this increase was absent in TRPV1 knockout mice compared with wild-type mice (Pacher et al. 2004).

It bears mentioning that the influence of cannabinoids on hemodynamic parameters is different in conscious animals. Unlike the three-phase changes described in anesthetized rats, anandamide elicited the first two phases (i.e. transient depressor and pressor responses) in conscious rats, but not the prolonged hypotension and bradycardia (Lake et al. 1997). This might be explained by the anesthetic agent, urethane, which attenuated the sympathetic suppression of CB1 (Kurz et al. 2009), or the relatively high resting sympathetic tone in anesthetized animals, which renders the hypotensive action of cannabinoids more evident (Carruba et al. 1987).

In humans, marijuana use and cannabinoid agents (sativex and nabilone) were associated with an acute acceleration of heart rate that usually peaks at 10 to 30 min after smoking (Karschner et al. 2011; Lile et al. 2011). This is regarded as an important biomarker of cannabinoid use (Zuurman et al. 2009). The CB1 antagonist rimonabant ameliorated the tachycardia caused by cannabis use (Huestis et al. 2007). Marijuana use also caused hypotension and dizziness in standing position (Mathew et al. 2003), which was attenuated by rimonabant (Gorelick et al. 2006). An *in vitro* study demonstrated that CB1 receptor activation by anandamide dilates human vessels by stimulating endothelial nitric oxide release (Bilfinger et al. 1998).

3.7.2. Atherosclerosis

Manipulation of cannabinoid receptors (CB2 receptor activation and CB1 receptor inhibition) might also limit atherosclerotic progression, as suggested by animal studies. The anti-atherosclerotic effects of CB2 receptors might be due, at least in part, to its anti-inflammatory actions. Steffens et al. detected CB2 expression in atherosclerotic plaques within human coronary arteries and mouse aorta, but not in regions free of atherosclerotic lesions. They also reported that THC, at a non-psychiatric dose, ameliorated the progression of atherosclerosis, reduced macrophage content and migration within atherosclerotic plaques, and suppressed T cell activation in apolipoprotein E-deficient mice, a common model of atherosclerosis. A CB2 receptor antagonist blocked all of these effects, indicating the protective role of CB2 receptors (Steffens et al. 2005). Similar effects were reported with WIN55, 212-2, which reduced atherosclerotic size, macrophage infiltration, adhesion molecule expression (i.e. vascular cellular adhesion molecule-1, intracellular adhesion molecule-1, and P-selectin), and expression of pro-inflammatory mediators (i.e. TNF-α, IL-6, and monocyte chemotactic protein 1) in a CB2-

dependent manner (Zhao et al. 2010; Zhao et al. 2010). Also, it attenuated oxidized low-density lipoprotein (oxLDL)-induced activation of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which in turn up-regulates pro-inflammatory factors (Zhao et al. 2010). In addition, CB2 receptor activation reversed TNF-α-induced proliferation of human coronary artery smooth muscle cells and the underlying MAPK pathway (Rajesh et al. 2008).

In contrast, evidence suggests that CB1 signaling contributes to the atherosclerotic process. First, CB1 antagonism reduced cholesterol deposition in macrophages. Jiang et al. reported suppression of PPARγ by the CB1 antagonist, AM251; PPARγ up-regulates fatty acid translocase/CD36 receptor, which mediates macrophage cholesterol influx, and down-regulates ATP-binding cassette protein A1, which mediates cholesterol efflux (Jiang et al. 2009). Sugamura et al. also showed that rimonabant reduced atherosclerotic lesions, and this was associated with increases in serum adiponectin, a protein involved in fatty acid degradation, and HDL cholesterol (Sugamura et al. 2010). Second, CB1 antagonism inhibits proliferation and migration of vascular smooth muscle cells. Reduced proliferation and migration of rimonabant-treated human coronary artery smooth muscle cells was observed in parallel to decreased ERK1/2 activation (Rajesh et al. 2008). Note, however, that clinical trials (STRADIVARIUS and AUDITOR) failed to demonstrate the ability of rimonabant to delay atherosclerotic progression (Nissen et al. 2008; O'Leary et al. 2011), but the STRADIVARIUS trial showed a favorable effect of rimonabant on HDL elevation and triglyceride decrease.

3.7.3. Ischemia/reperfusion injury

It is generally accepted that activation of cannabinoid receptors protects the heart against ischemia-reperfusion injury, primarily via CB2 receptor activation. Lagneux and Lamontagne first reported that the cardioprotective effects of lipopolysaccharide following

ischemia/reperfusion, namely infarct size reduction and improved myocardial contractility, were blocked by a CB2 receptor antagonist (Lagneux and Lamontagne 2001). Similar effects were reported subsequently for various endocannabinoids (anandamide, 2-AG and palmitoylethanolamide) (Lepicier et al. 2003; Li et al. 2013) and synthetic agonists (JWH-015 and ACEA) (Lepicier et al. 2003). A CB2 receptor antagonist completely abolished the reduction of infarct size by all of these compounds, whereas a CB1 receptor antagonist only partially blocked 2-AG-elicited effects (Lepicier et al. 2003). Possible mechanisms include CB2-dependent activation of Pl3K/Akt, p38/ERK1/2, and PKC, as well as inhibition of TNF-α and ROS (Lepicier et al. 2003; Li et al. 2013; Wang et al. 2012).

3.7.4. Cardiac hypertrophy and heart failure

As cardiac hypertrophy is a convergence point of risk factors for heart failure, there has been interest in the role of cannabinoid signaling. Anandamide and its metabolically-stable analog, R-methanandamide, suppressed hypertrophic indicators including cardiomyocyte enlargement and fetal gene activation (i.e. the brain natriuretic peptide gene) elicited by endothelin-1 in isolated neonatal rat ventricular myocytes (Lu et al. 2014). The ability of R-methanandamide to suppress myocyte enlargement and fetal gene activation was mediated by CB2 and CB1 receptors, respectively. Accordingly, a CB2-selective agonist, JWH-133, prevented only myocyte enlargement but not BNP gene activation. A CB1/CB2 dual agonist with limited brain penetration, CB-13, inhibited both hypertrophic indicators. CB-13 activated AMP-activated protein kinase (AMPK) and, in an AMPK-dependent manner, endothelial nitric oxide synthase (eNOS). Disruption of AMPK signaling, using compound C or shRNA knockdown, and eNOS inhibition using L-NIO abolished the anti-hypertrophic actions of CB-13 (Lu et al. 2014).

Liao et al. also queried a potential protective role of CB1 receptors by exposing CB1-deficient mice to transverse aortic constriction (TAC). CB1-deficient TAC mice exhibited higher mortality, more severe lung edema, and greater epinephrine and norepinephrine levels compared to wild-type TAC mice and CB1-deficient sham groups. They further demonstrated more advanced LV hypertrophy and contractile impairment, and this was associated with augmented MAPK (p38 and ERK1/2) activation. In wild-type TAC mice, CB1 agonism ameliorated lung edema, reduced plasma epinephrine and norepinephrine levels, and activated AMP-activated protein (AMPK) (Liao et al. 2012). CB1 receptor activation also suppressed MAPKs in cultured neonatal rat cardiac myocytes treated by isoproterenol (Liao et al. 2013). Results generated by Wagner et al. agreed with the protective role of CB1 in a rat model of post-infarction cardiac remodeling (Wagner et al. 2003). However, contradictory results suggest that CB1 antagonism improves cardiac performance. Mukhopadhyay et al. generated heart failure in mice using doxorubicin, an anti-cancer drug with severe cardiotoxicity. Cardiac performance-related parameters, including ejection fraction, cardiac output, contractility and apoptosis, deteriorated in response to doxorubicin, whereas CB1 antagonists rimonabant and AM281 were protective (Mukhopadhyay et al. 2007). More recently, Lin et al. showed that the LVH and fibrosis found in mouse model of uremic cardiomyopathy were attenuated by a CB1 antagonist. Also, in an *in vitro* model (indoxyl sulfate treated H9c2 cells), expression of fibrotic markers (collagen I, TGF-β and α-smooth muscle actin) was attenuated by a CB1 receptor antagonist or siRNA knockdown of CB1, vis-à-vis inhibition of Akt (Lin et al. 2015).

Regarding CB2 receptors, Weis et al. observed significant elevation of CB2 receptor expression in LV myocardium and endocannabinoids in blood circulation from patients with chronic heart failure, whereas CB1 receptor expression was down-regulated. These results

suggest activation of the endocannabinoid system during chronic heart failure, and in particular, of CB2 receptors (Weis et al. 2010). Increased CB2 receptor expression also occurs in patients with aortic stenosis and severe hypertrophic markers (Duerr et al. 2013). However, it is not clear whether this is a compensatory defense mechanism or a detrimental factor.

Collectively, extant evidence reveals contradictory effects of CB1 activation, and the role of CB2 remains unclear. This suggests that manipulation of cannabinoid signaling as a novel therapeutic approach to cardioprotection requires further investigation.

3.8. Endocannabinoid system and mitochondrial function

The endocannabinoid system is involved in various energy regulation processes, and it has been implicated in the regulation of appetite, body weight and diabetes (Horvath et al. 2012; C. Li et al. 2011). For example, CB1 antagonism reduced hepatic triglycerides, increased expression of genes involved in fatty acid oxidation, and improved insulin sensitivity (Tam et al. 2010). Mitochondria are therefore proposed as reasonable targets of the endocannabinoid system. Recently, studies on the endocannabinoid system and mitochondrial function have emerged. For example, Zaccagnino et al. observed reduced ATP synthesis without mitochondrial $\Delta\psi_m$ loss in isolated liver mitochondria treated by anandamide (Zaccagnino et al. 2011). Athanasiou et al. reported decreased mitochondrial $\Delta\psi_m$ and oxygen consumption in response to three cannabinoids: anandamide, THC, and the synthetic analog HU 210. Also, activities of ETC complexes I-III and cell viability were reduced, but only at concentrations higher than 10 μM, indicating concentration-dependent effects on mitochondrial function and integrity (Athanasiou et al. 2007). A few studies found cannabinoid receptor-independent effects of endocannabinoids on mitochondrial-dependent apoptosis by modulating the membrane fluidity, but again, at concentrations higher than 10 μM (Catanzaro et al. 2009; Siegmund et al. 2007). Nevertheless,

there is evidence to suggest that CB receptors regulate mitochondrial function, as discussed below.

3.8.1. Mitochondrial effects of CB1 receptors

CB1 receptors have been identified on mitochondrial membranes of mouse neuron cells, and account for approximately 15% of the total cellular amount (Benard et al. 2012). Fisar et al. reported that activation of CB1 receptors significantly reduced the activity of ETC complex I and II, but not complex IV in isolated mitochondria from pig brain (Fisar et al. 2014). CB1 receptor activation also reduced mitochondrial oxygen consumption and biogenesis parameters, such as mitochondrial mass and mitochondrial DNA amount, in mouse muscle and liver, as well as human white adipose tissue (Tedesco et al. 2010). In contrast, a CB1 antagonist increased fatty acid oxidation, reduced obesity in high-fat-diet mice (Jbilo et al. 2005), and prevented high-fat-induced cardiometabolic abnormalities in diabetic rats (Vijayakumar et al. 2012). This deregulation of mitochondrial function by CB1 receptors might be attributed to depressed p-AMPK and eNOS (Tedesco et al. 2010). In summary, existing evidence suggests that CB1 receptors negatively mediate mitochondrial biogenesis and fatty acid oxidation.

3.8.2. Mitochondrial effects of CB2 receptors

In contrast, studies suggest protective effects of CB2 receptor activation on mitochondrial performance. CB2 receptor activation slowed down neuron degeneration by preventing mitochondrial apoptotic pathways (Latini et al. 2014). In a rat model of myocardial ischemia/reperfusion, CB2 receptor activation by JWH133 inhibited mPT, mitochondrial membrane depolarization, cytochrome c release, and apoptosis, which were abolished by ERK1/2 inhibitor. Such effects were used to explain the cardioprotective actions of CB2 receptors against ischemia/reperfusion injury (Li et al. 2014; Li et al. 2013). Cardiac myocytes

exposed to the pro-hypertrophic agonist, endothelin-1, exhibited mitochondrial membrane depolarization in the presence of either palmitate or glucose as primary energy substrate, decreased mitochondrial bioenergetics and expression of genes related to fatty acid oxidation (i.e. PGC-1α, a driver of mitochondrial biogenesis, and CPT-1β, facilitator of fatty acid uptake); CB-13, a dual agonist of CB1 and CB2 receptors corrected these parameters in an AMPK-dependent manner, and it was speculated that this was due to actions mediated by CB2 rather than CB1 receptors (Lu et al. 2014). Contrary to CB1, CB2 receptor activation exhibits anti-obesity effects (Agudo et al. 2010; Verty et al. 2015); a possible mechanism is the stimulation of palmitate oxidation and related proteins, which is mediated by cAMP/PKA/sirtuin 1 (SIRT1)/PGC-1α signaling cascades (Zheng et al. 2013).

Taken in context with other studies on CB receptors, interventions that target CB/AMPK signaling might represent a novel therapeutic approach to address the multi-factorial problem of cardiovascular disease.

4. Regulation of endocannabinoid system components

Endocannabinoid and cannabinoid receptor levels may be altered by various factors such as stress, inflammation, high-fat diet, obesity, diabetes, and dietary fatty acid consumption.

4.1. Stress

Stress, depression, and anxiety are known to alter endocannabinoid levels. Memory retrieval in rats that underwent stressful training elicited an increase in 2-AG and a corresponding decrease in the activity of the 2-AG-degrading enzyme MAGL (Morena et al. 2015). A rat model of early life stress created by maternal deprivation also increased levels of endocannabinoid system components (CB1, CB2, TRPV1, GPR55, as well as endocannabinoid synthase and hydrolase) in frontal cortex and hippocampus of adolescent male and female rats

respectively (Marco et al. 2014). In addition, serum levels of endocannabinoids were evaluated in female patients with depression and anxiety. This study reported an increase in anandamide and 2-AG in patients with mild depression; however, 2-AG levels were markedly reduced in patients with advanced depression, and tended to decline with prolonged progression. Unlike the association of 2-AG levels with depression, anandamide was found to be negatively correlated with degree of anxiety (Hill et al. 2008).

4.2. Inflammation

Inflammatory conditions are often associated with elevated CB2 receptor expression. Multiple sclerosis patients exhibit higher CB2 expression in B cells, and higher anandamide levels in B cells, natural killer cells, and T cells (Sanchez Lopez et al. 2015). CB2 expression was also up-regulated in mouse models of, and humans, with colitis (Storr et al. 2009; Wright et al. 2005). Finally, anandamide, 2-AG, CB1 and CB2 receptors were detected in synovial membranes from patients with rheumatoid arthritis, but not healthy volunteers (Gui et al. 2015).

4.3. High-fat diet, obesity, and diabetes

High-fat diet, obesity, and diabetes are well-known conditions that involve altered levels of endocannabinoid system components. A marked increase in hepatic anandamide levels was detected in mice fed high-fat diets (60 en%) for three weeks, although the extent of increase declined after 14 weeks. This elevation of anandamide was associated with a reduction in FAAH activity (Osei-Hyiaman et al. 2005). In contrast, no changes in anandamide and 2-AG were observed in rats fed high-saturated fat diets (palm oil-rich diets, 38 en%) for one week (Artmann et al. 2008). Nevertheless, obese patients exhibit higher endocannabinoid levels in visceral fat and serum (Matias et al. 2006). Engeli et al. reported that compared to lean female subjects, obese females exhibited 35% and 52% increases in circulating anandamide and 2-AG

respectively, and a reduction of FAAH expression in adipose tissue (Engeli et al. 2005). Cote et al. observed a positive correlation between plasma 2-AG level and body mass index, intra-abdominal adiposity and fasting insulin level in males. However, a negative correlation was found between anandamide and intra-abdominal adiposity (Cote et al. 2007). Annuzzi et al. assessed the levels of endocannabinoids in subcutaneous adipose tissue, and found increased anandamide and decreased 2-AG in patients with both obesity and type 2 diabetes, but not in non-diabetic obese patients (Annuzzi et al. 2010). Increased FAAH and MAGL were detected in various adipose tissues (subcutaneous abdominal, visceral, and epididymal) in obese rats with or without diabetes. However, no changes in FAAH and MAGL were found in obese humans (Cable et al. 2014). The interaction between obesity and endocannabinoid levels remains unclear, and existing evidence appear to be controversial. However, endocannabinoid levels are seemingly not influenced solely by high-saturated fat diets; perhaps other mediators implicated in diabetes, such as leptin and insulin, may be key players (Matias et al. 2006). Indeed, intravenous injection of leptin significantly reduced anandamide and 2-AG levels in hypothalamus of normal rats and obese mice (Di Marzo et al. 2001). Insulin treatment decreased anandamide and 2-AG levels in healthy adipocytes, but not in insulin-resistant adipocytes (D'Eon et al. 2008).

4.4. Dietary consumption of polyunsaturated fatty acids (PUFA)

Anandamide and 2-AG are derived from arachidonic acid, an omega-6 PUFA. Thus, endocannabinoid levels may be modified by diets that affect the arachidonic acid content in tissue phospholipids. Indeed, increasing dietary linoleic acid (omega-6) (from 1 en% to 8 en%) elevated anandamide and 2-AG levels in mouse liver and resulted in weight gain, although total dietary fat remained unchanged (Alvheim et al. 2012). Also, the effects of omega-3 PUFA on

endocannabinoid levels were evaluated in mice brain. Watanabe et al. found that an omega-3 PUFA-deficient diet significantly increased 2-AG content in brain, whereas short-term consumption of a docosahexaenoic acid (DHA)-rich diet reduced the arachidonic acid content in phospholipids and brain 2-AG levels (Watanabe et al. 2003). These results were consistent with the findings of Wood et al., which showed that fish oil supplementation for 2 weeks is sufficient to affect fatty acid composition by enhancing DHA and eicosapentaenoic acid (EPA; omega-3 PUFA) levels, and down-regulated arachidonic acid and anandamide content in mouse brain and plasma (Wood et al. 2010). In addition, a human study investigated the effects of an omega-3-rich diet on endocannabinoid levels in obese men. In this study, krill powder, which contains 61.8% krill oil (omega-3-rich oil), was provided to mildly obese men for 24 weeks. After 24 weeks, plasma levels of anandamide and its analogues plmitoylethanolamide and oleoylethanolamide were significantly reduced, as were triglycerides, but no weight loss was observed (Berge et al. 2013). Although more studies are needed, the existing evidence implies that dietary fatty acid composition influences endocannabinoid levels by manipulating omega-3/omega-6 balance, which in turn regulates the level of arachidonic acid.

5. Conclusions

There is significant interest in manipulation of the endocannabinoid system as a therapeutic approach to treat disorders such as metabolic syndrome, inflammatory and neuropathic pain, and multiple sclerosis (Hosking and Zajicek 2008; Palazuelos et al. 2006). Unfortunately, therapeutic use of cannabinoids is impeded by psychotropic side effects including dysphoria, memory impairment, reduced concentration, disorientation, motor incoordination, and possibly addiction. This undesirable psychoactivity is mediated by central CB1 receptors (Hosking and Zajicek 2008; Howlett et al. 2002; Kunos et al. 2009; Piomelli 2003), so alternate strategies like using

CB2-selective agonists and/or peripherally-restricted CB1/CB2 dual agonists have been proposed (Gertsch et al. 2008; Hosking and Zajicek 2008; Kunos et al. 2009; Palazuelos et al. 2006). Collectively, the numerous studies on cannabinoid and CB receptor-mediated effects suggest that interventions that target cannabinoid signaling might represent a novel therapeutic approach to address multiple disease states and conditions.

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Table 1. Cannabinoid receptor agonists.

Cannabinoid agonists	Description	Ki for CB1 (nM)	Ki for CB2 (nM)	References
2-AG	CB1 and CB2 agonist	472	1400	(Stella et al. 1997)
ACEA	CB1-selective agonist	1.4	3100	(Hillard et al. 1999)
AM1241	CB2-selective agonist	280	3.4	(Ibrahim et al. 2003)
Anandamide	CB1 and CB2 agonist	89	371	(Pertwee 1999)
CB13	Peripherally-restricted CB1 and CB2 agonist	6.1	27.9	(Dziadulewicz et al. 2007)
CP55940	CB1 and CB2 agonist	3.72	2.55	(Felder et al. 1995)
HU-210	CB1 and CB2 agonist	0.061	0.52	(Felder et al. 1995)
JWH015	CB1 and CB2 agonist	383	13.8	(Showalter et al. 1996)
JWH133	CB2-selective agonist	677	3.4	(Huffman et al. 1999)
R-methanandamide	CB1 and CB2 agonist	20	815	(Abadji et al. 1994; Khanolkar et al. 1996),
THC	CB1 and CB2 agonist	53.3	75.3	(Felder et al. 1995)
WIN55, 212-2	CB1 and CB2 agonist	3.3	62.3	(Felder et al. 1995)

Table 2. Cannabinoid receptor antagonists.

Cannabinoid antagonists	Description	Ki for CB1 (nM)	Ki for CB2 (nM)	References
AM251	CB1-selective antagonist	7.5	2290	(Lan, Liu, et al. 1999)
AM281	CB1-selective antagonist	12	4200	(Lan, Gatley, et al. 1999)
AM630	CB2-selective antagonist	5200	31.2	(Ross et al. 1999)
SR141716	CB1-selective antagonist	2	>1000	(Rinaldi-Carmona et al. 1995)
SR144528	CB2-selective antagonist	400	0.6	(Rinaldi-Carmona et al. 1998)
AM6545	Peripherally-restricted CB1-selective antagonist	1.7	523	(Cluny et al. 2010)

Figure 1. Synthesis and degradation of 2-AG and anandamide.

Adapted with permission from (El Manira and Kyriakatos 2010).

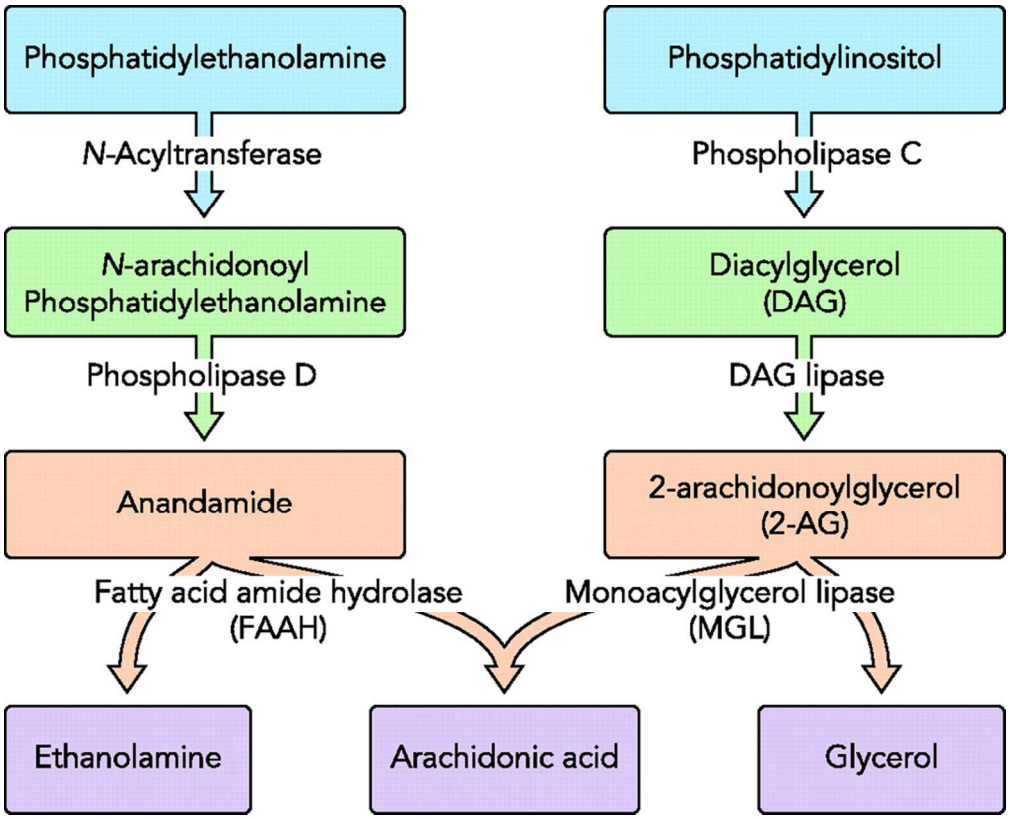

Figure 1. Synthesis and degradation of 2-AG and anandamide. Adapted with permission from (El Manira & Kyriakatos, 2010).

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