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Role of the Endocannabinoid System in Diabetes and Diabetic Complications

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Complete List of Authors:	Gruden, Gabriella; University of Turin, Medical Sciences Barutta, Federica; University of Turin, Medical Sciences Kunos, George; National Institutes of Health, National Institute on Alcohol Abuse and Alcoholism Pacher, Pal; National institutes of Health,
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Professor Cherry L Wainwright
Director, Institute for Health & Wellbeing Research
The Robert Gordon University
Riverside EastGarthdee Road
Aberdeen AB10 7GJ
Scotland, UK

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Dear Editor, Professor Wainwright,

Please find attached our invited review paper entitled “Role of the Endocannabinoid System in Diabetes and Diabetic Complications” by G. Gruden, F. Barutta, G. Kunos and P. Pacher for submission to the themed issue “Endocannabinoids: Synthesis and Function”. We hope that it will be a valuable contribution to the special issue.

Kind regards

Pal

Pál Pacher M.D., Ph.D., F.A.H.A., F.A.C.C.
Chief, Section on Oxidative Stress Tissue Injury,
Laboratory of Physiological Studies,
National Institutes of Health/NIAAA,
5625 Fishers Lane, MSC-9413,
Bethesda, Maryland 20892-9413, USA.
Phone: (301)443-4830;
Fax: (301)480-0257;
E-mail: pacher@mail.nih.gov

BJP invited review in "Endocannabinoids: Synthesis and Function"

Role of the Endocannabinoid System in Diabetes and Diabetic Complications

G. Gruden[1], F. Barutta[1], G. Kunos[2], P. Pacher[2]

[1]Laboratory of Diabetic Nephropathy, Department of Medical Sciences, University of Turin, Italy

[2]Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland, USA

Correspondence:

Pál Pacher M.D.,Ph.D.,F.A.H.A.,F.A.C.C.

Laboratory of Physiological Studies,

National Institutes of Health/NIAAA, 5625 Fishers Lane, MSC-9413,

Bethesda, Maryland 20892-9413, USA.

Phone:(301)443-4830;Fax:(301)480-0257; E-mail: pacher@mail.nih.gov

SUMMARY

An overactive endocannabinoid system(ECS) is a key factor in the development of diabetes, as it promotes energy intake and storage, alters both glucose and lipid metabolism, and has pro-apoptotic effects on pancreatic β cells. In addition, compelling evidence from preclinical studies indicates that the ECS also influences diabetes-induced oxidative stress, inflammation, fibrosis, and subsequent tissue injury in target organs for diabetic complications. In this review, we provide an update on the contribution of the ECS to the pathogenesis of diabetes and diabetic microvascular (retinopathy, nephropathy, and neuropathy) and cardiovascular complications. The therapeutic potential of targeting the ECS is also discussed.

Abbreviations: ECS-endocannabinoid system; AEA-anandamide;2-AG-2-arachidonoylglycerol; T2DM-type 2 diabetes mellitus;ROS/RNS-reactive oxygen/nitrogen species;DN-diabetic nephropathy;CB1/2R-cannabinoid receptor 1/2;MAPK-mitogen activated protein kinase;DNR-diabetic neuropathy.

INTRODUCTION

The major psychoactive component of Cannabis sativa, delta9-tetra-hydrocannabinol (THC), was identified 50 years ago. Since then, great effort has been directed to identifying the endogenous compounds whose biological actions are mimicked by THC and to clarify their role in various physiological and pathological processes. The endogenous cannabinoid system (ECS) comprises the endocannabinoids(ECs), the enzymes that regulate their production and degradation, and the receptors through which they signal. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the most studied ECs, are bioactive lipid mediators produced from cell membrane phospholipids. ECs are synthesized "on demand", AEA predominantly via hydrolysis of N-arachidonoylphosphatidylethanolamine by a phospholipase D, and 2-AG from diacylglycerol by diacylglycerol lipase, although parallel biosynthetic pathways also exist. Once synthesized, AEA or 2-AG are immediately released to target their receptors and then rapidly degraded by fatty acid amide hydrolase or monoacylglycerol lipase, respectively. The effects of ECs are mediated primarily by the $G_{i/o}$-coupled cannabinoid receptor 1 or 2(CB1R/CB2R), with the possible involvement of additional receptors, such as GPR-55. AEA signals predominantly via CB1R, while 2-AG is a full agonist at both CB1R and CB2R. Receptor activation results in a variety of biochemical responses, including inhibition of voltage-gated Ca^{++} channels and adenylate cyclase activity, leading to lower cAMP levels, as well as activation of K^{+} channels, phospholipases, and mitogen-activated protein kinase(MAPK) pathways, the latter via G protein-independent mechanisms(Howlett *et al*., 2010; Horvath *et al.,* 2012).

CB1R are expressed at very high levels in the central nervous system, whereas CB2R are predominantly found in immune, inflammatory, and hematopoietic cells(Pacher and Mechoulam, 2012). However, these receptors are present in several other cell types and the ECS has been implicated in a growing number of pathophysiologic processes. Thus, pharmacological modulation of the ECS emerges as a promising therapeutic strategy in a variety of pathological conditions,

including neurodegenerative, cardiovascular, gastrointestinal, liver, and renal diseases(Pacher and Kunos, 2006, 2013). Here we provide a brief overview of emerging evidence suggesting an important role of the ECS in the pathogenesis of type-2 diabetes (T2DM) and its chronic complications. The therapeutic potential of targeting the ECS in diabetes and diabetic complications will also be discussed.

DIABETES AND DIABETIC COMPLICATIONS

Diabetes mellitus affects 382 million people worldwide and this number is expected to rise to 592 million by 2035. The diabetes pandemic has been attributed to the growing prevalence of obesity, a major risk factor for T2DM. It has been estimated that almost 80% of T2DM cases could be prevented by adequate control of body weight. Diabetes is the seventh leading cause of death in the United States and both macrovascular and microvascular complications are the major cause of morbidity and mortality in diabetic patients. People with diabetes are two to six times more likely to develop macrovascular complications. Nearly half of all diabetic patients develop diabetic retinopathy and diabetes is the leading cause of blindness in adults, being responsible for 10,000 new cases of blindness every year in the United States alone. Diabetic nephropathy affects ~30% of patients with diabetes and diabetes is known to account for over 50% of all patients receiving renal transplants in the United States. About 60% of non-traumatic lower-limb amputations among people aged 20 years or older occur in people with diabetes and diabetic neuropathy is a major underlying cause(International Diabetes Federation, 2014).

Intervention studies have convincingly demonstrated that hyperglycemia is a major pathogenic factor for diabetic complications. The underlying mechanisms are not fully understood; however, formation of advanced glycation end products, activation of the polyol, the hexosamine and the protein kinase C pathways have been implicated. Oxidative stress through formation of both reactive oxygen and nitrogen(ROS/RNS) species is a common upstream event in the activation of

these deleterious metabolic/signalling pathways. Furthermore, inflammatory processes orchestrated by infiltrating monocytes/macrophages also contribute to target organ damage (Forbes and Cooper, 2013).

THE ROLE OF THE ECS IN THE PATHOGENESIS OF T2DM

Both insulin resistance in peripheral tissues and a relative deficiency in insulin secretion by islet β cells are key components in the development of T2DM. Studies performed in the last two decades have highlighted the central role of the ECS in the development of obesity and its deleterious effects on both glucose and lipid metabolism that can contribute to the development of insulin-resistance and T2DM. The well-established role of the ECS in metabolism has been recently reviewed(Silvestri and Di Marzo, 2013) and will only be briefly summarized. Recent emerging data suggest that the ECS also contributes to beta cell loss in T2DM by modulating inflammatory and cell death processes. These novel findings which may open an entirely new avenue to target the ECS in T2DM will be highlighted and discussed.

ECS in obesity and insulin resistance

In the CNS, activation of CB1R enhances food intake by modulating the activity of hypothalamic neurons and, subsequently, the release of orexigenic and anorexigenic neuropeptides. Furthermore, CB1R signalling affects reward and reinforcement circuits in the mesolimbic system, leading to a preference for highly palatable food. CB1R is also present in peripheral organs important in the control of metabolism and activate anabolic pathways, favoring energy storage. In white adipocytes, CB1R activation increases de novo fatty acid synthesis, enhances triglyceride accumulation and reduces lipolysis, whereas in brown adipose tissue CB1R counteracts the uncoupling of respiration from from ATP production. Furthermore, CB1R increases hepatic lipogenesis and drives defective oxidative metabolism through impaired mitochondrial oxidative phosphorylation in skeletal

muscle(Silvestri and Di Marzo, 2013;Boon *et al*., 2014;Kunos and Tam, 2011). The ECS has thus been proposed to be "part of a thrifty phenotype selected to cope with food shortage and make the best out of periods of plenty"(Di Marzo, 2012).

In abdominal obesity, the ECS is generally up-regulated in both central and peripheral tissues, as indicated by high EC levels and/or CB1R overexpression. The exact underlying mechanisms are unclear; however, ECs are lipid mediators and their biosynthesis can be directly influenced by dietary fat intake. This hyperactive ECS can contribute to further fat accumulation by enhancing food intake as well as by favoring lipogenesis and reducing energy expenditure in peripheral organs(Silvestri and Di Marzo, 2013;Kunos and Tam, 2011;Tedesco et al. 2010;Blüher *et al*., 2006;Silvestri *et al*., 2011). Consistently, both pharmacological and genetic CB1R blockade reduces body weight in animal models of obesity(Kunos and Tam, 2011). The effect of CB1R inhibition on food intake is transient and weight loss occurs predominantly through blockade of peripheral CB1R. However, recent data suggests that central ECS also controls peripheral energy metabolism(O'Hare *et al*., 2011). As visceral adiposity is a major determinant of insulin resistance, it is not surprising that ECS over-activity favors the development of obesity-associated metabolic abnormalities.

Emerging data suggest that a deranged ECS has also direct deleterious effects on insulin sensitivity and glucose metabolism independently of weight gain. In adipose tissue, activation of the ECS enhances glucose uptake to increase energy storage in the form of de novo synthesized lipids, downregulates adiponectin thereby affecting insulin-sensitivity at distant organs, and may favor local inflammation(Ge *et al*., 2013;Murumalla *et al*., 2011). In skeletal muscle, CB1R interferes with glucose uptake by inhibiting signalling pathways activated by insulin, including those required for plasma-membrane translocation of glucose transporters. In the liver, activation of hepatic CB1R can reduce systemic insulin sensitivity independently from body weight. Indeed, mice that express CB1R exclusively on hepatocytes remain lean when fed a high fat diet, but they develop hepatic and systemic insulin resistance, whereas mice with hepatocyte-specific CB1R deletion become obese, but remain insulin-sensitive(Liu *et al*., 2012). Several mechanisms may underlie these

findings: hepatic CB1R activation reduces insulin clearance by reducing the hepatic expression of the insulin-degrading enzyme and inhibits insulin signaling through IRS1 and akt-2, resulting in increased hepatic glucose production due primarily to increased glycogenolysis(Liu *et al*., 2012). Furthermore, CB1R activation induces ER stress resulting in elevated hepatic levels of long-chain ceramides that in turn inhibit insulin signaling(Cinar *et al*., 2014). Collectively, these data provide strong evidence that a deranged ECS due to conditions leading to obesity, such as a high fat diet, may then contribute to further fat accumulation and insulin-resistance through excess CB1R activity and thus set the stage for the development of T2DM.

There is relatively little knowledge on the role of CB2R in the control of metabolic processes; however, recent studies suggest CB2R may affect inflammatory aspects of both obesity and T2DM. Surprisingly, CB2R agonists potentiated obesity-associated inflammation, insulin resistance, and hepatic steatosis and CB2R deficiency improved insulin sensitivity(Deveaux *et al*., 2009;Agudo *et al.,* 2010). Furthermore, CB2R overexpression in the brain induces hyperglycaemia and a lean phenotype in adult mice(Romero-Zerbo *et al*., 2012). However, these studies need additional confirmation with improved CB2 selective ligands (particularly given the potent anti-inflammatory role of CB2 agonists reported in numerous pathological disease models(Pacher and Mechoulam, 2012).

ECS and pancreatic beta cells

Data on the expression of ECS components in pancreatic islet cells are contradictory and vary among species; however, most studies agree that beta cells express both ECs and CB1R and that CBR1 activation enhances insulin release(Horváth *et al*., 2012;Malenczyk *et al*., 2013). Recent studies have explored the possibility that the ECS may favor the development of T2DM by inducing beta cell apoptosis. ZDF rats are a valuable animal model to address this issue because they replicate T2DM natural history. Indeed, young ZDF rats are insulin resistant and normoglycemic, while older ZDF become hyperglycemic because of progressive beta cell failure. In this model,

ibipinabant, a global CB1R antagonist, attenuates beta cell loss independently of its effects on body weight(Rohrbach *et al*., 2012). Furthermore, a peripherally restricted CB1R antagonist JD5037 delays the progression of T2DM and beta cell function loss, confirming that EC acting through peripheral CB1R can contribute to beta cell failure(Jourdan *et al*., 2013).

In beta cells insulin itself positively regulates beta-cell survival and resistance to apoptosis in an autocrine manner and recent in vitro studies suggest that CB1R forms a heteromeric complex with the insulin receptor and thus inhibits insulin signaling by blocking insulin receptor kinase activity. This causes reduced phosphorylation of the pro-apoptotic Bad, thereby causing beta cell death(Kim *et al*., 2012). Although these in vitro findings suggest that EC may induce beta cell death by acting directly on beta cells, a recent study has convincingly shown that beta cell failure in adult ZDF rats is not associated with CB1R signaling in beta cells, but rather in proinflammatory macrophages infiltrating pancreatic islets. Specifically, CB1R activation in macrophages induced activation of the Nlrp3-ASC inflammasome, resulting in the proteolytic activation and release of IL-1β and IL-18, which act as paracrine signals to induce beta cell apoptosis(Jourdan *et al*., 2013). The dominant role of macrophages in progressive beta cell death does not, however, exclude the possibility that high glucose acting on beta cells may trigger the inflammatory process by inducing IL-1β and MCP-1 release and thus macrophage infiltration. Data on CB2R expression in beta cells are controversial; however, given the key role of the CB2R in inhibiting inflammatory processes, it would be of interest to explore the potential protective role of signalling through this receptor in inflammatory cell-mediated beta cell death.

Intervention studies in humans and future perspectives

Clinical trials in obese and T2DM patients have proven the efficacy of the global CB1R inverse agonist rimonabant in reducing body weight and waist circumference and ameliorating both lipid and glucose control. Based on these promising data, rimonabant was licensed in over 50 countries worldwide for the treatment of obesity. However, the drug was subsequently withdrawn from the

market because of an increased risk of psychiatric adverse events, such as anxiety, depression and suicidal ideation, and the therapeutic development of this class of compounds was discontinued(Christensen *et al*., 2007).

More recently, peripherally restricted CB1R antagonists that poorly cross the blood-brain barrier and are thus devoid of centrally mediated psychiatric side effects have been developed to assess if peripheral CB1R inhibition preserves the metabolic benefit of global CB1R blockade. A proof of principle study by Tam *et al.*(2010) demonstrated that treatment of diet-induced obese mice with the peripherally restricted neutral CB1R antagonist AM6545 improved glucose tolerance, insulin sensitivity, plasma lipid profile, and also reversed fatty liver, though it was less effective than rimonabant in reducing body weight and it did not affect caloric intake. Subsequent studies have shown that a highly potent, selective and brain impermeable CB1R inverse agonist, JD5037, is even more effective in improving metabolic parameters in rodent models of obesity/diabetes, has hypophagic effects by reversing leptin resistance(Tam *et al*., 2012), abolishes obesity-induced hepatic insulin-resistance(Cinar *et al*., 2014), and preserves beta cell function in ZDF rats(Jourdan *et al*., 2013). These results raise hope that CB1R blockade may still be a viable option to combat dysmetabolism and JD5037 is currently undergoing toxicology screening and may move to clinical testing in the near future.

DIABETIC NEPHROPATHY

Diabetic nephropathy(DN) is a leading cause of end-stage renal failure and significantly enhances the cardiovascular risk of diabetic patients. The complication is characterized by both increased glomerular permeability to proteins and a relentless decline in renal functions. Structural changes comprise podocyte abnormalities, including nephrin loss, mesangial expansion, and tubulo-interstitial fibrosis. It is well established that oxidative stress, inflammation, and fibrogenesis play a pivotal role in the development and progression of DN(Forbes and Cooper, 2013). Given the pro-

oxidative, pro-inflammatory and pro-fibrotic effects of CB1R signalling and the opposing effects of signalling through CB2R, there is growing interest on the potential role of the ECS in the pathogenesis of DN.

A full ECS is present within the normal kidney. In healthy animals, CB1R is expressed by endothelial cells of the renal arteries and weakly by podocytes and tubular epithelial cells (TEC); while CB2R is strongly expressed by podocytes. This pattern of expression changes profoundly in diabetes. CB1R is overexpressed by podocytes in animal models of in both type 1(T1DM) and 2 diabetes(Barutta *et al*., 2010; Tam *et al*., 2012; Jourdan *et al.,* 2014). On the contrary, there is a deficiency of 2-AG, the main CB2R ligand, in the renal cortex from mice with early STZ-induced diabetes and podocyte CB2R expression is markedly downregulated in human biopsies from patients with advanced DN(Barutta *et al*., 2011). Taken together these data indicate that a shift in the balance of EC signalling to favor the deleterious effects of CB1R over the protective effects of CB2R occurs. It is likely that both hyperglycemia and hypertension are important determinants of these alterations as in cultured podocytes exposure to high glucose was shown to increase CB1R expression(Nam *et al*., 2012), while mechanical stress, mimicking glomerular capillary hypertension, downregulates CB2R(Barutta *et al*., 2014). Moreover, proteinuria may lower constitutive tubular CB2R expression in advanced DN as exposure of tubular epithelial cells to albumin downregulates CB2R expression(Jenkin *et al*., 2013).

Intervention studies in animal models of DN have uncovered a potentially important role of the ECS in the pathogenesis of DN. The first evidence was provided in murine models of the metabolic syndrome. Treatment with rimonabant prevented proteinuria, ameliorated renal function, and reduced the glomerular damage in obese Zucker diabetic fatty rats and improved both albumin-creatinine ratio and glomerulosclerosis in JCR:LA-cp rats(Janiak *et al*., 2007;Russell *et al*., 2010). More recently, a study performed in db/db mice, a model of T2DM, has shown that rimonabant markedly decreases urinary albumin excretion and mesangial expansion and suppresses synthesis of profibrotic and proinflammatory cytokines(Nam *et al*., 2012). However, CB1R blockade also

significantly improved insulin resistance and lipid profile in these animals, and the observed renoprotetion may be due, at least in part, to improvement of metabolic abnormalities. Convincing proof for the direct role of CB1R in the development of DN arose from a study performed in STZ-induced diabetes, a model of T1DM, in which protective metabolic effects of CB1R blockade cannot confound outcomes. In this model, treatment with the selective CB1R reverse agonist AM251 significantly reduced albuminuria and prevented downregulation of nephrin and podocin, suggesting that enhanced podocyte CB1R signalling may contribute to the development of albuminuria by lowering the expression of podocyte proteins crucial to maintaining glomerular permselectivity(Barutta *et al*., 2010). There was no effect of CB1R blockade on markers of renal fibrosis and it is unclear whether the differential effect on fibrogenesis observed in animal models of T1DM versus T2DM reflects true differences in underlying mechanisms or whether it is animal strain-related. In vitro, CB1R activation is profibrotic, as it mediates the effects of high glucose both in inducing podocyte collagen overexpression(Nam *et al*., 2012) and promoting mesangial cell apoptosis(Lim *et al*., 2011); however, it is still controversial if CB1R are present in mesangial cells in vivo(Barutta *et al*., 2014). A recent study using ZDF rats(Jourdan et al., 2014) provided additional mechanistic insight on the role of CB1R in the pathogenesis of diabetic nephropathy. This study demonstrated that peripheral CB1R blockade was not only effectively in preventing the characteristic hallmarks/symptoms of diabetic nephropathy(albuminuria, reduced glomerular filtration, activation of renin-angiotensin system, oxidative/nitrative stress, podocyte loss and increased CB1R expression in glomeruli), but could also reverse these changes after they developed. This study also provided evidence that the enhanced CB1R signaling in diabetic kidneys promotes upregulation of the local angiotensine II receptor-NADPH oxidase signalling promoting ROS generation in podocytes and cell death(Jourdan et al., 2014).

Recent studies have highlighted an important protective role for CB2R in DN. In STZ-induced diabetes, activation of CB2R by the selective CB2 agonist AM1241 reduced albuminuria, glomerular monocyte accrual, and nephrin downregulation(Barutta *et al*., 2011). Conversely,

knocking-down CB2R worsened slit diaphragm protein downregulation, proteinuria, overexpression of extracellular matrix components, mesangial matrix expansion, monocyte infiltration, and renal function loss in diabetic mice(Barutta *et al*., 2014). CB2R activation reduced MCP-1 signalling, whereas CB2R deficiency markedly increased the expression of the MCP-1 receptor CCR2 in the renal cortex, as well as in both cultured podocytes and monocytes(Montecucco *et al*., 2008;Barutta *et al*., 2011;Barutta *et al*., 2014). By lowering CCR2 expression in monocytes, CB2R agonists may reduce the recruitment of inflammatory cells that can contribute to renal injury through the release of ROS, toxic products and cytokines. On the other hand, CB2R-induced CCR2 downregulation on podocytes may prevent the direct deleterious effects of MCP-1 on this cell type, including nephrin downregulation(Giunti *et al*., 2010;Tarabra *et al*., 2009). Of interest, recent experiments employing adoptive transfer of bone marrow have clarified that the worsening of DN in CB2R-deficient mice is mainly due to CB2R loss on podocytes rather than on monocytes(Barutta *et al*., 2014).

Studies performed in experimental cisplatin-induced nephropathy have shown that both CB1R blockade and CB2R activation reduce tissue injury, cell death, and interrelated inflammation and oxidative/nitrosative stress(Mukhopadhyay *et al*., 2010a and b). This suggests that CB1R and CB2R also have opposing effects on tubular epithelial cells that may be of relevance in the pathogenesis of diabetes-induced tubulo-interstitial injury. In keeping with this notion, palmitic acid, that promotes of tubulointerstitial damage in T2DM, induces CB1R expression in cultured proximal tubular epithelial cells and CB1R mediates palmitic acid-induced endoplasmic reticulum stress and apoptosis. Furthermore, AEA causes proximal tubular epithelial cell hypertrophy and this effect is reduced by CB1R antagonists and enhanced by CB2R antagonists. Although, tubular hypertrophy initially leads to increased capacity of the proximal tubules to reabsorb albumin, an increase in albumin reabsorption can activate fibrotic cytokines, contributing to tubulo-interstitial injury(Jenkin *et al*., 2012).

Collectively these data suggest a beneficial effect of both CB1R blockade and CB2R activation in DN. This is of significant therapeutic relevance since 20% of patients with incipient DN still progress to overt nephropathy despite optimal treatment, and there is increasing need for novel therapeutic strategies. Further studies are required to establish the therapeutic potential of peripheral restricted CB1R antagonists or CB2R agonists in DN and to find out whether the addition of these compounds to current DN treatment protocols results in extra benefit.

DIABETIC NEUROPATHY

Diabetic neuropathy (DNR) affects as many as 60% of patients with long-standing diabetes. Distal symmetrical polyneuropathy (DSP), the most common type of DNR, is due to axon degeneration secondary to both metabolic abnormalities and injury of endoneural microvessels. Almost a third of patients with DSP describe burning, electric, or stabbing pain(allodynia/hyperalgesia)(Peltier *et al*., 2014) and there is considerable interest in the possibility of exploiting the anti-nociceptive properties of the ECS for therapeutic gain.

Treatment with CB1R agonists has anti-nociceptive effects in STZ-induced diabetes(Horváth *et al*., 2012;Vera *et al*., 2012). Peripherally-restricted CB1R agonists, devoid of central side effects, are likely to be equally effective as analgesia is predominantly due to activation of CB1R on peripheral nociceptors(Agarwal *et al*., 2007). However, as discussed above, CB1R activation contributes to the development of T2DM and its complications in addition to deletorious cardiovascular effects, which is a major obstacle to their therapeutic use(Pacher and Kunos, 2013). CB2R agonists also exert anti-nociceptive effects in diabetic mice, which appear to be predominantly related to inhibition of microglia-driven inflammation(Vincenzi *et al*., 2013). In contrast to CB1R agonists, CB2R agonists do not have unwanted central side effects and appear to be protective in most of diabetic complications. However, CB2R agonism has been reported to have deleterious effects on metabolism(Deveaux *et al*., 2009;Agudo et al., 2010), which is still a matter of debate and require

further clarification. Furthermore, positive results in animals do not imply efficacy in humans, as some mixed CB1/2R agonists have so far performed poorly in patients, despite efficacy in rodents (in part because of the metabolic and cardiovascular adverse effects attributable to CB1R stimulation). A clinical trial performed in 30 patients with painful DNR randomised to either Sativex, containing both THC and cannabidiol, or placebo, has failed to show any benefit of Sativex(Selvarajah *et al*., 2010), though depression was a major confounding factor during the study.

Besides the potential importance of the ECS as a therapeutic target in painful DNR, there is also evidence for its potential role in the pathogenesis of DNR, although the data are often conflicting. Expression of CB1R was found to be reduced in dorsal root ganglia of diabetic rats and CB1R activation attenuated neural damage and normalised neurite outgrowth in cells exposed to a high glucose milieu(Zhang *et al*., 2009). On the other hand, in vivo studies suggest that inhibition rather than activation of CB1R may be beneficial. In STZ-induced diabetes, treatment with rimonabant partially prevented loss of intraepidermal nerve fiber density and increased current perception threshold. These effects were paralleled by reduced skin capillary loss, increased blood flow, and diminished tissue TNF-alpha levels, suggesting that the observed effects may be related to the anti-inflammatory and vasoprotective properties of rimonabant(Liu *et al*., 2010). Furthermore, in diabetic mice, rimonabant attenuated mechanical allodynia, reduced oxidative stress in peripheral nerves, inhibited TNF-alpha overexpression in the spinal cord, and moderated NGF deficiency, suggesting that CB1R blockade interferes with mechanisms leading to nerve injury and favours nerve regeneration. Accordingly, the histological analysis of sciatic nerves showed a marked degeneration of myelinated fibers in diabetic mice that were reduced by rimonabant treatment(Comelli *et al*., 2010).

Taken together, the studies summarised above suggest that CB1R signalling enhances the inflammatory and oxidative processes leading to both neuronal and microvessel damage, in addition to having some neuroprotective and anti-nociceptive properties. Therefore, CB1R effects may vary

substantially in different experimental settings and species, which may underlie the conflicting data. Further research is required to reconcile controversies and to establish whether and what type of modulation of ECS activity is a feasible therapeutic strategy in DNR.

DIABETIC CARDIOMYOPATHY AND RETINOPATHY

Both major cannabinoid receptors as well as endocannabinoid synthetic and metabolising enzymes are expressed in the myocardium and vasculature. Based on preclinical studies, under normal physiological conditions the ECS appears to play only a very limited, if any, role in cardiovascular regulation. However, it emerges as an important player in triggering or promoting disease pathology/progression in cardiovascular disease(Pacher and Kunos, 2006). Similarly to the diabetic nephropathy discussed above, it appears that activation of CB1 and CB2 receptors have opposing consequences in various major cardiovascular pathologies. ECs acting via CB1R generally promote hypotension, bradycardia and negative inotropy via receptors located on sympathetic and parasympathetic nerve terminals, cardiomyocytes and endothelial cells(Pacher and Kunos, 2006). In addition, endocannabinoids through CB1 receptor-dependent/independent pathways may also promote ROS generation and activation of pro-apoptotic stress signalling pathways(e.g. p38 and JNK mitogen-activated protein kinases) in murine and human cardiomyocytes, endothelial and smooth muscle cells, and promote pro-fibrotic signalling in fibroblasts/myofibroblasts(Rajesh *et al*., 2010,2012;Mukhopadhyay *et al.,* 2010c;Tiyerili *et al.,* 2010). Emerging evidence also suggests that endocannabinoid activation of CB1 receptors promotes pro-inflammatory signalling in macrophages and enhance recruitment of various inflammatory cells to the site of insult, facilitating cardiovascular inflammation, vascular or myocardial remodelling and tissue injury(Steffens and Pacher, 2014). In agreement with this, ECs and CB1R have been implicated in the pathogenesis of cardiac dysfunction, cell death, and inflammation in various forms of shock, heart failure, and atherosclerosis(Pacher and Kunos, 2006). In contrast, activation of CB2R in immune cells

attenuates chemotaxis, adhesion of inflammatory cells to the activated endothelium, and activation of these immune cells. CB2R activation also attenuates endothelial cell activation and pro-inflammatory response, decreases smooth muscle proliferation, and may exert protective effects in cardiomyocytes(Pacher and Steffens, 2012). These effects are responsible for the benefits of CB2R agonists reported in myocardial, cerebral and other models of ischemic/reperfusion injury(Pacher and Hasko, 2008). However, the role of CB2 in cardiomyocytes requires additional confirmation in light of concern with the specificity of the commercially available CB2R antibodies(Pacher and Steffens, 2012). Endocannabinoids may also exert numerous CB1/2R independent effects (e.g. vasodilation/vasoconstriction, anti-inflammatory/pro-inflammatory, etc.) in the cardiovascular or other organ systems via degradation to arachidonic acid metabolites or through putative novel cannabinoid or other (e.g. TRPV1) receptors) depending on the context and concentration/dose used(Pacher and Kunos, 2013;Stanley and O'Sullivan, 2014).

Although diabetes is a well-recognized risk factor for cardiovascular disease and heart failure, the mechanisms of the development and progression of diabetic cardiomyopathy, which involve complex interplay of oxidative/nitrative stress with metabolic, proinflammatory and cell death pathways, are still not completely understood(Varga *et al.*, 2014).

Using a mouse model of type 1 diabetic cardiomyopathy, Rajesh *et al.*,(2012) investigated the role of EC-CB1R signaling in myocardial dysfunction, inflammation, remodelling and cell death. They found increased levels of anandamide and increased CB1R expression in diabetic hearts, accompanied by enhanced accumulation of advanced glycation end products(AGEs), oxidative/nitrative stress, inflammation, cell death and fibrosis. This also paralleled with enhanced angiotensin II type 1 receptors-p47(phox) NADPH oxidase signalling, β-myosin heavy chain isozyme switch, decreased expression of sarcoplasmic/endoplasmic reticulum Ca(2+)-ATPase (SERCA2a) and both diastolic and systolic cardiac dysfunction(Rajesh *et al.*, 2012). These pathological processes were markedly attenuated by CB1R blockade with globally acting CB1R antagonists or by genetic deletion of CB1R. These effects were glucose-independent, as CB1

inhibition had no effect on the elevated blood glucose levels following destruction of pancreatic beta cells by multiple injections of streptozotocin, yet CB1R blockade not only prevented but also reversed the pathological remodelling and diabetic cardiac dysfunction in this type I diabetes model. In db/db mice, chronic CB1R inhibition attenuated myocardial fibrosis and remodelling, similar to its earlier described beneficial effects in diabetic nephropathy(Nam *et al.*, 2012). CB1 receptor inhibition also improved cardiac function and remodelling after experimental myocardial infarction and metabolic syndrome by mechanisms similar to those described above Slavic *et al.,*(2013). Furthermore, acute and chronic systemic CB1 cannabinoid receptor blockade improved blood pressure regulation and metabolic profile in an angiotensin II-dependent hypertensive(mRen2)27 rat model(Schaich *et al.,* 2014).

Supporting the pathological function of an overactive ECS in cardimetabolic diseases, increased plasma levels of AEA and 2-AG were strongly correlated with adverse coronary circulatory events or impaired coronary endothelial function in human obese subjects(Quercioli *et al.,* 2011; Pacher and Kunos, 2013). These studies even suggested that plasma EC levels be considered as biomarkers of cardiovascular risk in obese populations.

Collectively, the above studies strongly suggest that activation of CB1R by endocannabinoids contributes to the pathogenesis of diabetic cardiovascular dysfunction by facilitating AT1R expression/AT1R-NADPH oxidase-ROS signallin, MAPK activation, AGE accumulation, oxidative/nitrative stress, inflammation and fibrosis. These mechanisms are also critical in the development of other microvascular complications of diabetes (e.g. diabetic nephropathy (discussed in the earlier parts) and retinopathy(El-Remessy *et al.,* 2011; Horvath *et al.*, 2012), as indicated by the beneficial effects of CB1R inhibition or genetic deletion.

Thus, inhibition of peripheral CB1R with a new generation of peripherally restricted antagonists/inverse agonists may represent a promising strategy in the treatment of diabetic cardiovascular complications.

CONCLUSION AND PERSPECTIVES

CB1R blockade is beneficial in animal models of obesity and metabolic syndrome, and these findings have been confirmed in humans. Furthermore, recent preclinical studies suggest that "peripherally restricted" CB1R antagonists may represent a novel therapeutic strategy to minimize or avoid neuropsychiatric liability while retaining metabolic efficacy in obesity, insulin resistance, and beta cell loss. These new compounds deserve further development and clinical testing as they might have a significant clinical impact. Alternative strategies to counteract EC over-activity would be to develop drugs that lower EC levels through modulating their biosynthesis and/or degradation, or to develop dietary interventions that would lower the abundance of endocannabinoid precursors. Future studies will clarify if these new approaches are feasible.

Cannabinoid-based therapies may also protect against diabetic complications. The opposing effects of CB1R and CB2R on inflammation, oxidative stress and fibrogenesis likely explain the beneficial effects of CB1R blockade and CB2R activation in the setting of diabetic complications. Although data on the functional consequences of CB1R gene polymorphism are still lacking, an association between a common CB1R polymorphism and the presence of both nephropathy and retinopathy has been recently reported in T2DM patients(Buraczynska et al., 2014). Thus, second generation CB1R antagonists may have promise in the treatment of diabetic complications.

Regarding the therapeutic potential of CB2R agonists, it is important to emphasize that their effect on worsening insulin-resistance, if confirmed by other studies using more specific ligands, may hamper their use in the treatment of T2DM complications. It is important to note that the CB2R agonists used in studies so far may not have been entirely specific, particularly at high doses, and may have induced unwanted, CB1R-mediated effects(Pacher and Mechoulam, 2011). Therefore, it is very important to develop more selective CB2R agonists.

In conclusion, modulation of the ECS in diabetes and diabetic complications with peripherally restricted synthetic CB1 antagonists and/or CB2 agonists holds therapeutic promise. Furthermore,

marijuana-derived substances such as cannabidiol, which does not interact with classical cannabinoid receptors but has been reported to exert beneficial effects in diabetes and diabetic complications, may also have therapeutic utility(Horvath and Pacher, 2012), the discussion of which is beyond the scope of this review.

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AUTHOR CONTRIBUTION

All authors contributed to writing and editing the manuscript.

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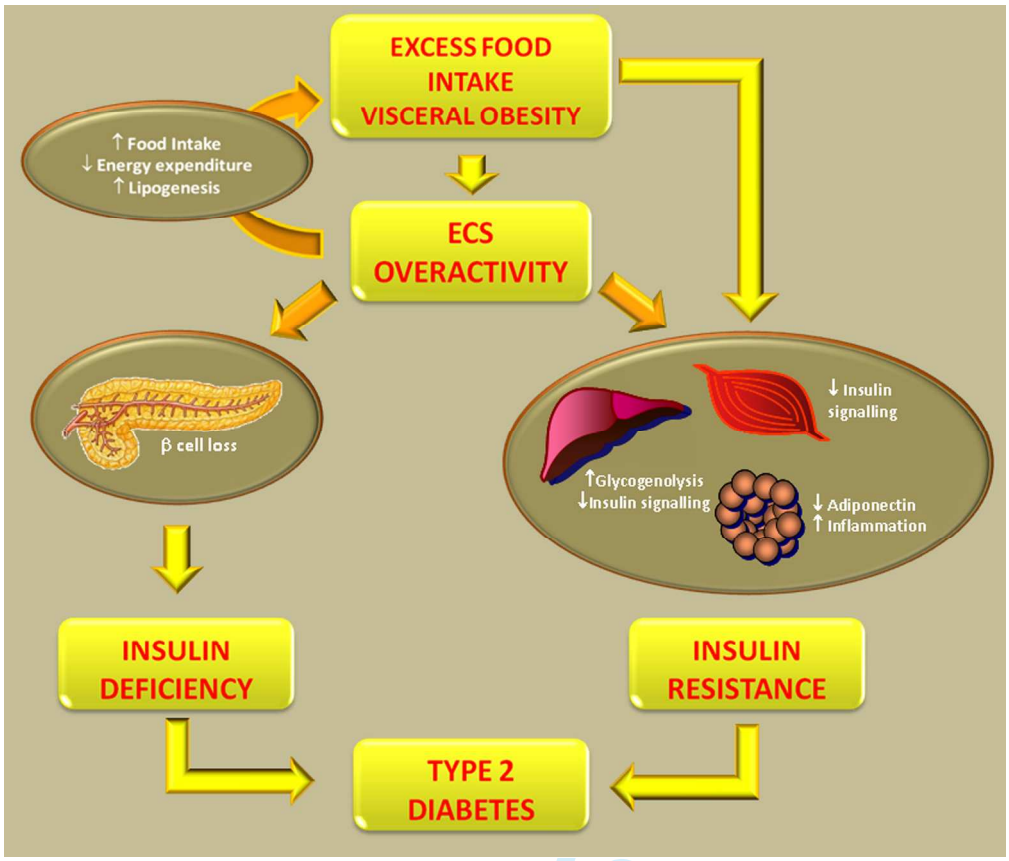
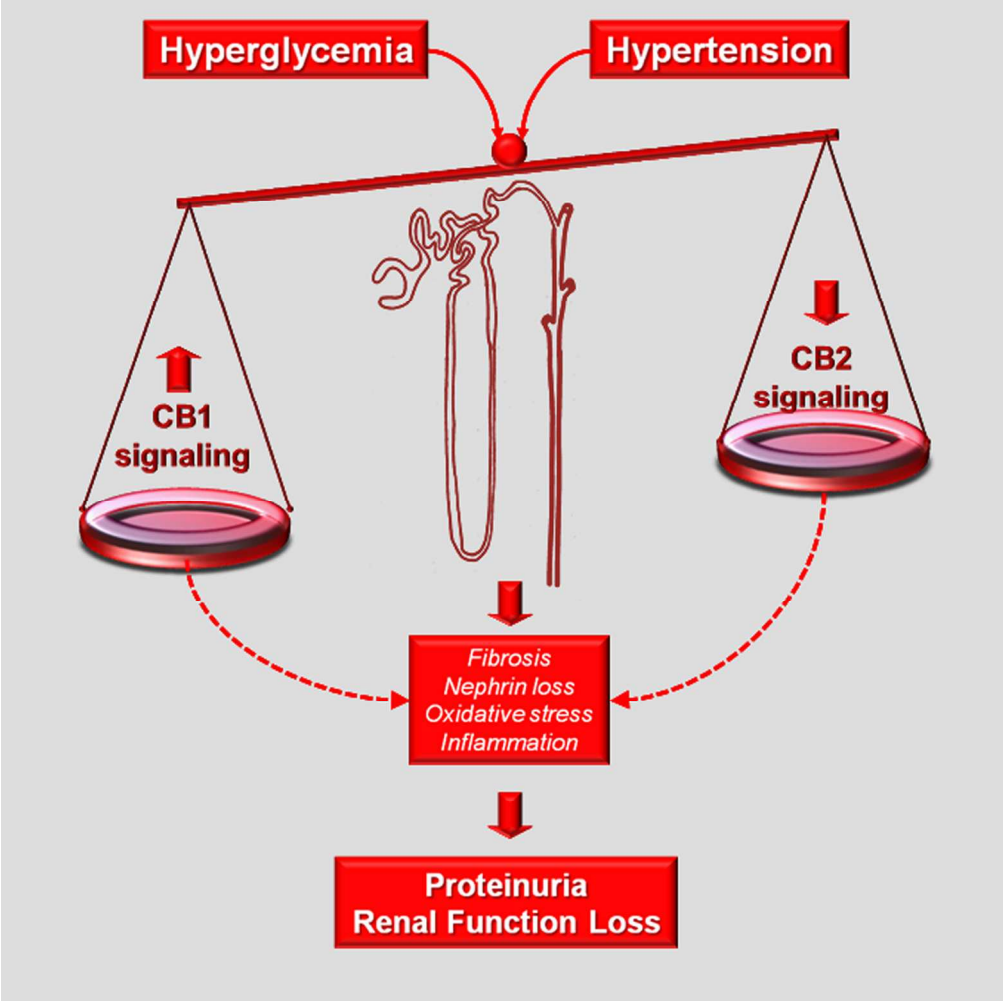
FIGURE LEGENDS

Figure 1: Role of the Endocannabinoid System (ECS) in the Development of Type 2 Diabetes. Excess food intake and obesity enhance the ECS tone. A hyperactive ECS further contributes to visceral fat accumulation and obesity by reducing energy expenditure and by enhancing both food intake and lipogenesis. Therefore, the ECS is involved in the development of obesity-dependent insulin resistance. Moreover, an overactive ECS has direct deleterious effects on insulin sensitivity independent of weight gain in peripheral organ of metabolism (liver, adipose tissue, skeletal muscle). Finally, the ECS indirectly contribute to beta cell failure through activation of the Nlrp3-ASC inflammasome in infiltrating macrophages, resulting in beta cell apoptosis. Both insulin resistance and relative insulin deficiency lead to the development of type 2 diabetes.

Figure 2: Opposing Effects of CB1R and CB2R in Diabetic Nephropathy
The CB1R has deleterious pro-oxidative and pro-inflammatory effects, while opposing protective effects are induced by CB2R activation. In diabetes hyperglycemia and hypertension alter the balance between CB1R and CB2R signalling as CB1R expression is enhanced, while CB2R is downregulated. This unbalance favors oxidative stress, inflammatory, and profibrotic processes and contributes to development of proteinuria by enhancing nephrin loss and of renal function loss by exacerbating fibrogenesis in both the mesangium and tubulo-interstitium.

Figure 3: Role of the Endocannabinoid-CB1R Signalling in Diabetic Cardiovascular Complications. Hyperglycemia and hyperlipidemia associated with diabetes promotes increased reactive oxygen and nitrogen species (ROS/RNS) generation in endothelium, vascular smooth muscle and cardiomyocytes, induces stress signalling, pro-fibrotic changes and cell death in the myocardial cells, as well as leads to activation and recruitment of inflammatory cells with consequent pro-inflammatory response. Hyperglycemia also directly or inderectly leads to enhanced EC-CB1 receptor signalling, which in turn amplifies these pathological processes facilitating tissue injury, cardiovascular dysfunction and eventualy development of diabetic cardiovascular complications such as cardiomyopathy, nephropathy, retinopathy and enhanced atherosclerosis.


EXCESS FOOD INTAKE VISCERAL OBESITY
↑ Food Intake
↓ Energy expenditure
↑ Lipogenesis
ECS OVERACTIVITY
β cell loss
↓ Insulin signalling
↑Glycogenolysis
↓Insulin signalling
↓ Adiponectin
↑ Inflammation
INSULIN DEFICIENCY
INSULIN RESISTANCE
TYPE 2 DIABETES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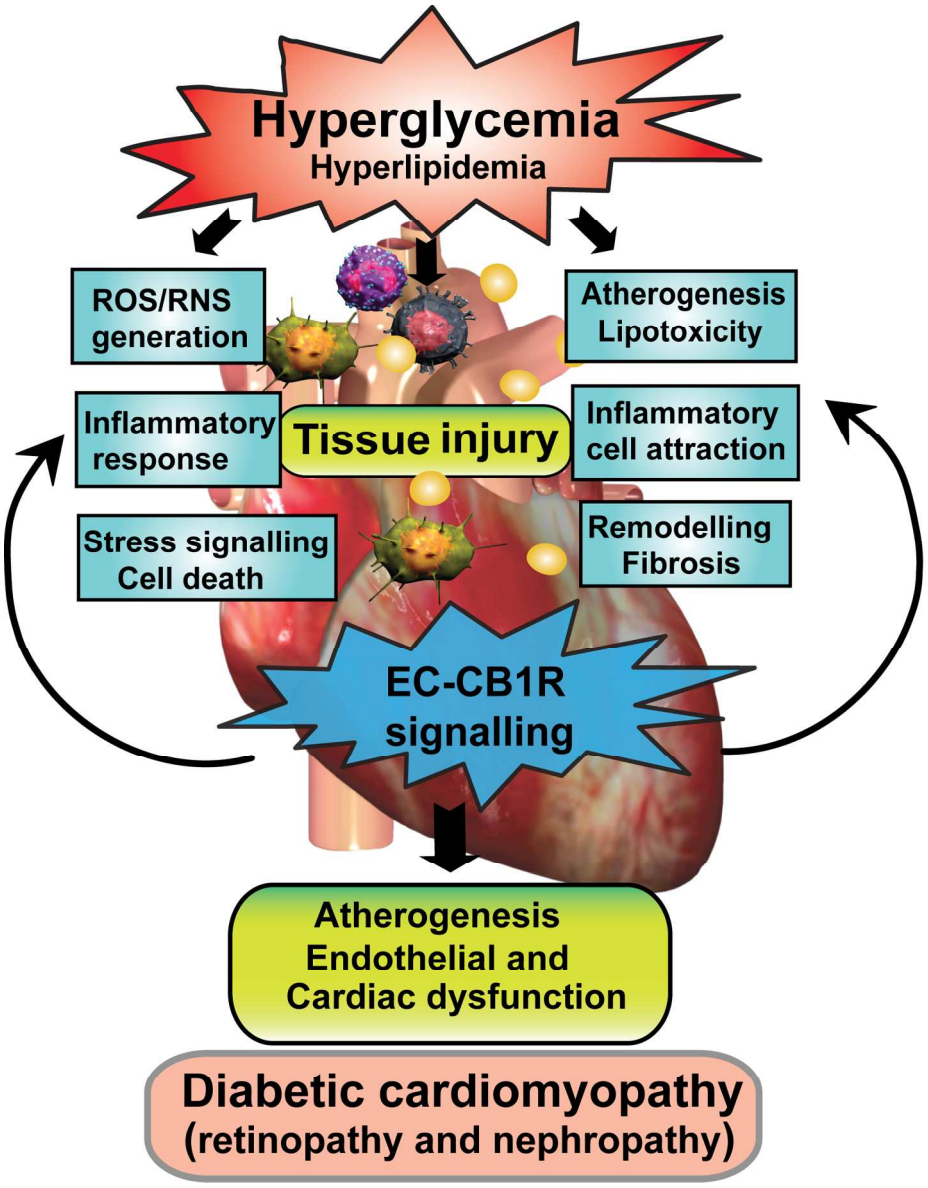
Hypertension
CB1 signaling
CB2 signaling
Fibrosis
Nephrin loss
Oxidative stress
Inflammation
Proteinuria
Renal Function Loss



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