
Laboratory of Physiologic Studies

George Kunos, MD, PhD, *Chief*

The common theme in the Laboratory of Physiologic Studies is the use of *in vivo* animal models of human pathological states relevant to alcohol use disorders.

The **Section on Neuroendocrinology** conducts studies on the biology of endogenous cannabinoids and their receptors. Work focuses on the role of the endocannabinoid system in the control of energy homeostasis, including energy intake and peripheral energy metabolism, and its role in cardiovascular regulation. The work on energy intake aims to explore the role of endocannabinoids in the neuroendocrine control of appetite, including the appetitive aspects of ethanol drinking behavior. Endocannabinoids are also involved in the regulation of peripheral metabolism, and research in this section was first to identify the liver as a primary target of the effect of endocannabinoids on lipid metabolism and steatosis, induced either by high-fat diets or chronic alcohol intake.



Other studies highlight the role of endocannabinoid in cardiovascular regulation, particularly the regulation of cardiac contractile function in hypertension, and in the hypotensive state associated with liver cirrhosis. Work in the Section has also uncovered a putative novel endothelial receptor for endocannabinoids that mediates vasodilation, and selective ligands for this receptor have been developed.

The primary interest of the **Section on Liver Biology** is the immunological aspects and molecular pathogenesis of liver diseases. There are two main directions: 1) the role of innate immunity in liver injury and repair, and 2) and the role of the STAT family of transcription factors in mediating cytokine-induced liver injury and repair.

Alcohol consumption, nonalcoholic steatohepatitis (NASH), and viral hepatitis are three major causes of chronic liver injury leading to liver fibrosis, cirrhosis, and liver cancer. Increasing evidence suggests that immune cells play an important role in the pathogenesis of liver disease. The liver predominantly expresses innate immunity through the involvement of innate immune cells, including macrophages (i.e., Kupffer cells), natural killer (NK) cells, and natural killer T (NKT) cells. These cells are important as the first line of defense against infection and are poised to quickly respond to potential attacks by any pathogen in the absence of antigen recognition. Interestingly, in liver injury and repair, innate immunity is activated. Hence, this laboratory is investigating the immunologic mechanisms of liver injury and repair, focusing on innate immunity in liver disorders such as fatty liver and liver fibrosis, as well as in liver regeneration.

Another focus is the role of signal transducer and activator of transcription 3 (STAT3), a cytokine-activated transcription factor, in liver pathology. The group uses several murine models to study the roles IL-6, IL-22 and STAT3 in the development and progression of fatty liver diseases, liver injury, and repair. They have identified the important role of IL-6 in protecting

against liver injury in NASH, fatty liver transplantation, and T cell hepatitis. Currently, they are exploring the role of STAT3 in these models by using STAT3 conditional knock out mice. They are also interested in studying the roles of other members of the STAT family in the above disorders.

The **Section on Oxidative Stress Tissue Injury** examines the role of oxidative-nitrosative stress and consequent poly(ADP-ribose) polymerase (PARP) activation in various forms of cardiovascular pathologies, including those that are induced by chronic alcohol intake or develop as a consequence of aging. The preferred approach is to use a combination of state-of-the-art in vivo methodologies to analyze hemodynamic functions in combination with cell and molecular biological methods to study the expression and function of cellular proteins involved in tissue injury and inflammation, or acting as anti-inflammatory, protective and tissue repair mechanisms. As an example for the latter, the role of endocannabinoids as anti-inflammatory mediators acting via CB₂ cannabinoid receptors in peripheral tissues is analyzed in ischemia-reperfusion models of tissue injury.

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The Biology and Functions of the Endocannabinoid System

Endocannabinoids are lipid-like mediators discovered in the 1990s that bind to cannabinoid receptors to trigger effects similar to those of marijuana. Endocannabinoids and their receptors are expressed at high levels in the brain, but are also present in many peripheral tissues. The Section on Neuroendocrinology, Laboratory of Physiologic Studies, is focused on exploring the role of the endocannabinoid system in the regulation of appetitive functions, including alcohol drinking behavior, in lipid metabolism with emphasis on their role in obesity, and in cardiovascular functions. Identification of as-yet undefined cannabinoid receptors and enzymes involved in the biosynthesis of endocannabinoids are also current goals.

The role of the endocannabinoid system in obesity/metabolic syndrome

Prompted by the well known effect of marijuana to increase appetite, studies in the late 1990's have shown that the cannabinoid CB₁ receptor antagonist, rimonabant, reduced food intake in rodents. Although this suggested that endocannabinoids may tonically stimulate appetite, the inverse agonist properties of rimonabant could also explain these findings. Our laboratory produced the first definitive evidence for the orexigenic role of endocannabinoids by demonstrating that mice deficient in CB₁ receptors have reduced food intake following temporary food deprivation (Di Marzo et al., *Nature* 2001). Additional findings indicated that endocannabinoids in the hypothalamus are downregulated by the anorexigenic hormone, leptin, and upregulated in rodents lacking leptin or a functional leptin receptor, suggesting that endocannabinoids are part of the leptin-regulated neural circuitry controlling appetite (Di Marzo et al., 2001) and may be involved in some forms of obesity. Recent electrophysiological studies by others identified lateral hypothalamic neurons containing melanin concentrating hormone as a site of leptin/endocannabinoid interaction in the brain (Jo et al., *Neuron* 48:1055, 2005).

CB₁ deficient mice are resistant to obesity and the associated hormonal/metabolic alterations induced by a high-fat diet even though their total caloric intake does not differ from the intake of wild-type mice that do become obese on the same diet. This indicated that reduced food intake cannot account for the role of endocannabinoids in the control of body weight, which must include direct effects on peripheral metabolism. Our findings revealed that CB₁ receptors and endocannabinoids are present in the liver where their activation promotes lipogenesis and

inhibits lipid oxidation, thus contributing to the development of high-fat diet-induced fatty liver (Osei-Hyiaman et al., *J Clin Invest* 2005). To determine the contribution of hepatic CB₁ receptors to the other components of the metabolic syndrome (increased adiposity, dyslipidemia, insulin and leptin resistance), we analyzed the effects of a high-fat diet in mice with selective genetic ablation of hepatic CB₁ receptors (LCB₁^{-/-} mice) in comparison with wild-type (wt) and global CB₁ knockout (CB₁^{-/-}) mice. LCB₁^{-/-} mice fed a high-fat diet developed a similar degree of obesity as wt mice but, similar to CB₁^{-/-} mice, had less steatosis, hyperglycemia, dyslipidemia, and insulin and leptin resistance than wt mice fed a high-fat diet. Thus, activation of hepatic CB₁ receptors by endocannabinoids contributes to diet-induced steatosis and the associated hormonal/metabolic changes, but not to the increased adiposity (Osei-Hyiaman et al., *J Clin Invest* 2008).

The role of the hepatic endocannabinoid system in alcoholic fatty liver

Similar to high-fat diet-induced hepatic steatosis, chronic ethanol intake leads to fatty liver due to increased hepatic lipogenesis and decreased fat elimination. In a separate study we reported that the steatosis induced in mice by a low-fat, liquid ethanol diet is attenuated by concurrent blockade of CB₁ receptors. Both CB₁^{-/-} and LCB₁^{-/-} mice are resistant to ethanol-induced steatosis and increases in lipogenic gene expression, and have increased CPT-1 activity, which, unlike in controls, is not reduced by ethanol feeding. Ethanol feeding increases the expression of CB₁ receptors in hepatocytes and upregulates 2-AG and its biosynthetic enzyme diacylglycerol lipase-β in hepatic stellate cells. In control, but not in CB₁ receptor-deficient hepatocytes, coculture with stellate cells from ethanol-fed mice results in upregulation of CB₁ receptors and lipogenic gene expression. These findings suggest that paracrine activation of hepatic CB₁ receptors by stellate cell-derived 2-AG mediates ethanol-induced steatosis by increasing lipogenesis and decreasing fatty acid oxidation (Jeong et al., *Cell Metab*, 2008). They also indicate a novel role of stellate cells in the control of hepatic fat metabolism and steatosis.

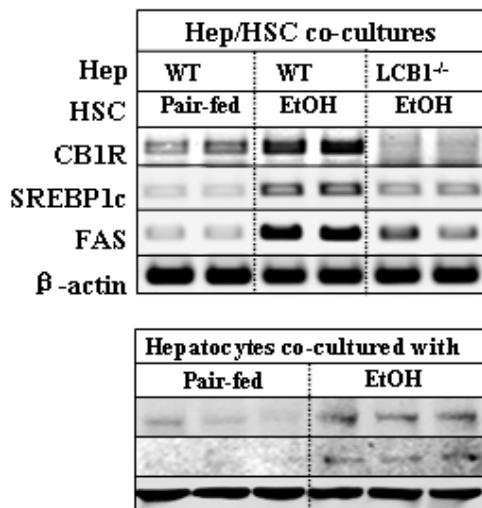


Fig. 1. Co-culture with stellate cells from EtOH-fed mice leads to upregulation of CB₁, SREBP1c and FAS mRNA (A) and protein (B) in control but not in CB₁-deficient (LCB₁^{-/-}) hepatocytes (from Jeong et al., *Cell Metab*. 2008)

Ongoing studies are aimed at identifying the molecular targets of CB₁ receptor activation in the liver, including AMP kinase and its upstream regulators. Furthermore, the relative role of peripheral versus central CB₁ receptors is explored by examining the role of hepatic innervation and hormonal signals such as adiponectin in the effects of cannabinoids on liver fat metabolism. These studies also involve the use of mice with liver-specific knock-in on a CB₁ knockout background. Additional studies are aimed at exploring the mechanism by which endocannabinoids induce and CB₁ blockade reverses leptin-resistance.

Endocannabinoids and cardiovascular functions.

Cannabinoids and their endogenous counterparts have vasodilator and cardiodepressor effects *in vitro* and elicit hypotension *in vivo*. We have therefore explored the role of endocannabinoids in regulating cardiovascular variables under physiologic and pathologic conditions. In earlier studies we have established that the hypotension associated with various forms of shock, including hemorrhagic, endotoxic and cardiogenic shock, are partly mediated by macrophage-derived endogenous anandamide acting at CB₁-like cannabinoid receptors. Advanced liver cirrhosis is characterized by generalized vasodilation and hypotension. The possible role of endocannabinoids in this condition was suggested by the elevated blood levels of bacterial endotoxin in late-stage cirrhosis. Evidence for this has been provided by the findings that a) rimonabant reverses the hypotension and mesenteric vasodilation in two models of experimental cirrhosis in rats, b) macrophages from cirrhotic rats and humans have elevated anandamide content and can elicit hypotension when injected into normal test rats, and c) CB₁ receptors are upregulated in hepatic vascular endothelial cells isolated from cirrhotic vs. non-cirrhotic human livers (Batkai et al., *Nature Med* 2001).

Cirrhosis is also associated with cardiac contractile dysfunction, termed ‘cirrhotic cardiomyopathy’. In a more recent study we found that rats with CCL₄-induced cirrhosis develop decreased cardiac contractility (Fig. 2), as documented through the use of the Millar pressure-volume microcatheter system, as well as low blood pressure and tachycardia. Bolus injection of a CB₁ receptor antagonist (AM251, 3 mg/kg i.p.) acutely increases blood pressure as well as both load-dependent and load-independent variables of systolic function (Fig. 2), whereas no such effects is observed in control rats. Furthermore, there is a selective, 2.7-fold increase in the myocardial content of anandamide, but not 2-AG, in the cirrhotic myocardium, whereas in the cirrhotic liver both endocannabinoids increased, with the increase in 2-AG content (6-fold) being more robust than the increase in anandamide (3.5-fold). There is no change in CB₁ expression in the cirrhotic vs normal myocardium. These findings suggest that activation of cardiac CB₁ receptors by endogenous anandamide contributes to the decreased cardiac contractility in cirrhosis (Batkai et al., *Am J Physiol Heart Circul Physiol*, 2007), whereas elevated levels of hepatic endocannabinoids may contribute to the increased fibrogenesis, as suggested by others (Teixeira-Clerc et al., *Nature Med* 12:671, 2006)

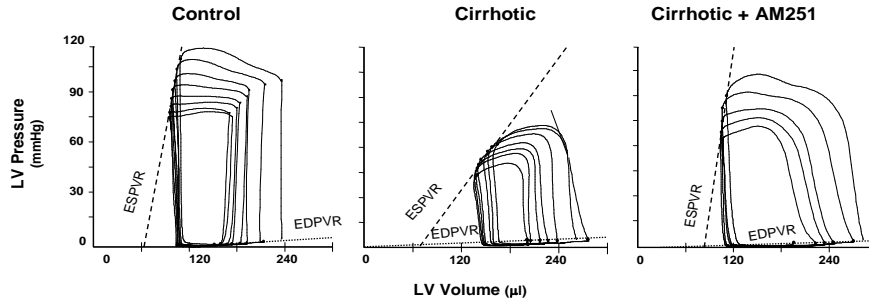


Fig. 2. Decreased cardiac contractility in cirrhosis reversed by CB₁ blockade. Intraventricular pressure-volume loops obtained at different preloads are shown. Note that the slope of the end-systolic P-V relation (ESPVR) is less steep in cirrhosis than in the other two groups.

Novel molecular components of the endocannabinoid system

Endocannabinoids and their plant-derived and synthetic analogs produce their effects via G protein-coupled cannabinoid receptors, of which two have been identified to date: CB₁, expressed at very high levels in the CNS but also present at lower levels in peripheral tissues, and CB₂, expressed predominantly in immune and hematopoietic cells. We have reported that the endothelium-dependent vasodilator effect of anandamide and certain atypical cannabinoids is mediated by a pertussis toxin-sensitive GPCR distinct from CB₁ or CB₂ (Begg et al., 2005). We have also reported that arachidonoyl-L-serine, a lipid-like substance identified in the brain, has vasodilator properties and is a likely endogenous ligand of this receptor (Milman et al., 2006).

In a recently completed study, we have analyzed the vasorelaxant effect of ARA-S in isolated vascular preparations and its effects on Ca²⁺-activated K⁺ currents in human embryonic kidney cells stably transfected with the α -subunit of the human, large conductance Ca⁺-activated K⁺ (BK_{Ca}) channel (HEK293hSlo cells). ARA-S caused relaxation of rat isolated, intact and denuded, small mesenteric arteries pre-constricted with phenylephrine, whereas it caused further contraction of vessels pre-constricted with KCl. Vasorelaxation by ARA-S was inhibited by 100 nM iberiotoxin. In HEK293hSlo cells, ARA-S and its enantiomer *N*-arachidonoyl-D-serine enhanced the whole cell outward K⁺ current with similar potency (pEC₅₀: 5.63 and 5.32, respectively). The potentiation was not mediated by ARA-S metabolites, stimulation of known cannabinoid receptors, G proteins, protein kinases or Ca²⁺-dependent processes; it was lost after patch excision or following membrane cholesterol depletion, but was restored after cholesterol reconstitution. BK_{Ca} currents were also enhanced by anandamide (AEA, pEC₅₀: 5.27) but inhibited by another endocannabinoid, virodhamine (pIC₅₀: 6.35), or by the synthetic cannabinoid O-1918, which blocks ARA-S-induced vasodilation (pIC₅₀: 6.59). We conclude that (i) endocannabinoid-like lipids directly modulate the activity of BK_{Ca} channels or a channel-associated component. (ii) This interaction does not involve cannabinoid receptors or cytosolic factors but is dependent on the presence of membrane cholesterol (iii) Direct BK_{Ca} channel activation likely contributes to the endothelium-independent component of ARA-S-induced mesenteric vasorelaxation. (iv) Depending on the structure of the head group, the effect on BK_{Ca} currents is either stimulatory or inhibitory. (v) O-1918 is a potent BK_{Ca} channel inhibitor (Godlewski et al., JPET 2008).

In vivo generation of the endocannabinoid anandamide from its membrane phospholipid precursor, N-arachidonoyl phosphatidyl ethanolamine (NAPE) is thought to occur through hydrolysis catalyzed by a NAPE-specific PLD. Recent evidence indicates, however, the existence of two additional, parallel pathways. One involves the sequential deacylation of NAPE by α,β -hydrolase 4 (Abhd4) and the subsequent cleavage of glycerophosphate to yield anandamide (Simon & Cravatt, *J Biol Chem* 281:26465, 2006), and the other one, recently identified in our laboratory (Liu et al., 2006), proceeds through PLC-catalyzed conversion of NAPE to phospho-anandamide, and its subsequent dephosphorylation by phosphatases, including PTPN22, to anandamide. In a recent study we found that in macrophages, the endotoxin-induced increase in anandamide synthesis proceeds uniquely through this PLC/phosphatase pathway, the other two pathways only contributing to basal tissue levels of anandamide (Liu et al., 2008). Ongoing studies are aimed at identifying additional phosphatases involved in the biosynthesis of anandamide through NAPE dephosphorylation.

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Hepatic Inflammation and Immunity

Alcohol consumption, nonalcoholic steatohepatitis, and viral hepatitis are three major causes of chronic liver injury leading to liver fibrosis, cirrhosis, and liver cancer. At present, the molecular and cellular mechanisms underlying liver injury and repair in these liver disorders are poorly understood. However, increasing evidence has suggested that immune cells play an important role in the pathogenesis of liver disease.

The hepatic immune system predominantly expresses innate immunity, whereby a large percentage of innate immune cells, including macrophages (i.e., Kupffer cells), natural killer (NK) cells, and natural killer T (NKT) cells are involved. These cells are important as the first line of defense against infection and are poised to quickly respond to potential attacks by any pathogen in the absence of antigen recognition via producing a variety of cytokines. Interestingly, in liver injury and repair, innate immunity is activated, followed by production of a wide variety of cytokines. Hence, our laboratory is investigating the immunologic mechanisms of liver injury and repair, focusing on innate immunity and cytokines in fatty liver, liver inflammation and fibrosis, as well as in liver regeneration.

Innate Immunity, Liver Injury, Fibrosis, and Repair

Jeong, Park, Wang, and Gao in collaboration with Tian, Kim, Lian, Gershwin

Our laboratory is actively studying: 1) the role of innate immune cells (NK/NKT cells) and cytokines [interferon (IFN)/ signal transducer and activator of transcription 1 (STAT1)] in liver injury, fibrosis, and regeneration; 2) the effects of ethanol on innate immunity in the liver.

Regardless of etiology, all forms of chronic liver injury lead to liver fibrosis accompanied by an excessive accumulation of extracellular matrix proteins, including collagen. Recent evidence suggests that liver fibrosis, and even cirrhosis, can be reversible. While activation of hepatic stellate cells has been known to play a central role in the development and progression of liver fibrosis, the clearance of hepatic stellate cells by apoptosis has been suggested to be a key step involved in reversing liver fibrosis. However, the factors responsible for hepatic stellate cell apoptosis during liver fibrosis remain largely unknown. Our recent findings indicated that NK cells play an important role in inducing hepatic stellate cell apoptosis during liver fibrosis. Using an *in vitro* culture model, we demonstrated that NK cells kill early-activated hepatic stellate cells,

but not quiescent or chronically activated stellate cells. Furthermore, this appeared to be due to the fact that early-activated hepatic stellate cells, but not quiescent or chronically-activated stellate cells, express high levels of the NK cell activating ligand, retinoic acid early inducible gene 1 (RAE1), which activates NK cell killing. RAE1 proteins were originally isolated from mouse embryonic carcinoma F9 cells treated with retinoic acid and later identified as the NKG2D ligand to activate NK cells (see Radaeva et al., 2006). Currently, we are exploring the roles of NKT cells in chronic liver injury and liver fibrosis. Our preliminary findings show that NKT cells play a diverse role in acute liver injury, but are depleted in chronic liver injury induced by carbon tetrachloride, suggesting that NKT cells may play a role in inhibiting the early stage of liver fibrosis but not the late stage of disease.

It is well documented that chronic alcohol consumption accelerates liver fibrosis in patients with hepatitis C virus (HCV) infection. Multiple mechanisms have been proposed to explore the underlying mechanisms mediating ethanol's effects. Our laboratory has demonstrated that chronic alcohol consumption attenuates the anti-fibrotic effects of innate immune cells (NK/IFN- γ), which could be an important mechanism contributing to alcohol acceleration of liver fibrosis in patients with chronic HCV infection (see Jeong et al., 2008).

Cytokines/STATs in Fatty Liver, Liver Inflammation, and Repair

Horiguchi, Miller, Lafdil, Wang, and Gao in collaboration with Kunos, Fu, Hennighausen, Pacher, Young.

Our laboratory has been also actively studying the roles of cytokines and their signaling pathways in liver diseases, focusing on the role of IL-6/STAT3 in fatty liver, liver inflammation and repair. We have previously demonstrated that IL-6 plays an important role in protecting against liver injury in several murine models of alcoholic liver injury, nonalcoholic fatty liver disease, fatty liver transplantation, and T cell hepatitis. It is believed that the action of IL-6 is mainly mediated via activation of signal transducer and activator of transcription 3 (STAT3).

By using immunohistochemistry analyses, we have demonstrated that phosphorylated STAT3 (STAT3 activation) are detected in hepatocytes, sinusoidal endothelial cells, bile duct-like cells, and inflammatory cells (macrophages, neutrophils, etc.) in human alcoholic cirrhotic livers (see Horiguchi et al., 2007). To understand the roles of STAT3 in alcoholic liver injury, we created liver-specific and macrophage/neutrophil-specific STAT3 knock out mice by crossing STAT3^{flox/flox} mice with albumin-promoter Cre transgenic mice and lysozyme M-promoter Cre transgenic mice, respectively. Compared with wild-type mice, feeding hepatocyte-specific STAT3 knock out mice with an ethanol-containing diet induced greater hepatic steatosis, hypertriglyceridemia, and hepatic expression of lipogenic genes (sterol regulatory element-binding protein, fatty acid synthase, acetyl-CoA carboxylase-1, and stearoyl-CoA desaturase 1), but less inflammation and lower expression of hepatic proinflammatory cytokines. In contrast, ethanol-fed macrophage/neutrophil-specific STAT3 knock out mice showed more hepatic inflammation, worse injury, and increased hepatic expression of proinflammatory cytokines compared with wild-type mice. Kupffer cells isolated from ethanol-fed hepatocyte-specific STAT3 knock out mice produced similar amounts of reactive oxygen species and tumor necrosis factor alpha, whereas Kupffer cells from macrophage/neutrophil-specific STAT3 knock out mice produced more reactive oxygen species and tumor necrosis factor alpha compared with wild-type controls. Our findings suggest that STAT3 regulates hepatic inflammation in a cell type-

dependent manner during alcoholic liver injury: STAT3 in hepatocytes promotes, whereas STAT3 in macrophages/Kupffer cells suppresses, inflammation. In addition, activation of hepatocellular STAT3 ameliorates alcoholic fatty liver via inhibition of sterol regulatory element-binding protein 1c expression (see Horiguchi et al., 2008). Currently, we are exploring the roles of endothelial cell- and macrophage/neutrophil-specific STAT3 in nonalcoholic fatty liver disease and liver regeneration.

In addition, we are also collaborating with Drs. George Kunos and Pal Pacher from NIAAA to investigate the role of the endocannabinoid system in alcoholic liver disease.

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Mechanisms of Oxidative-Nitrosative Stress and Inflammation-Induced Tissue Injury

Overwhelming evidence suggests that oxidative-nitrosative stress and inflammation are involved in essentially all major pathological processes affecting humans. The goal of the laboratory is to understand the cellular and molecular mechanisms underlying oxidative-nitrosative stress and inflammation-induced tissue injury using clinically relevant animal models of disease, and to identify novel therapeutic targets against these pathologies.

Oxidative-nitrosative stress and poly(ADP-ribose) polymerase in cardiovascular pathophysiology, ischemia/reperfusion injury and diabetic complications: cellular and molecular mechanisms.

Oxidative-nitrosative stress and consequent poly(ADP-ribose) polymerase (PARP) activation are key events in the development of endothelial and myocardial dysfunction in various models of cardiovascular injury and heart failure (ischemic, drug-induced and aging-associated; Fig. 1). Importantly, novel drug candidates targeting this pathway are entering or being evaluated in Phase II trials for a variety of critical care diseases associated with reperfusion injury and inflammation. These include, but are not limited to, ischemic stroke, acute respiratory distress syndrome, thoraco-abdominal aortic aneurism, repair surgery and the complications associated with cardiopulmonary bypass, and myocardial infarction as well as primary percutaneous coronary intervention.

We have demonstrated that peroxynitrite, a highly reactive oxidant formed from the reaction of nitric oxide and superoxide anion, is a key mediator of Doxorubicin (a widely-used chemotherapeutic drug)-induced cell death in cardiomyocytes and endothelial cells.

Diabetic vascular dysfunction is a major clinical problem which can lead to retinopathy, nephropathy, neuropathy and increased risk of stroke, hypertension and myocardial infarction. We have demonstrated that the aldose reductase inhibitor, fidarestat, counteracts diabetes-associated cataract formation, retinal oxidative-nitrosative stress, glial activation, and apoptosis. We have also described that the novel inosine analogue, INO-2002, protected against development of diabetes in mice. We have also shown that Adenosine_{A2A} receptor activation inhibited T helper 1 and T helper 2 cell development and effector functions and protected against the development of diabetes.

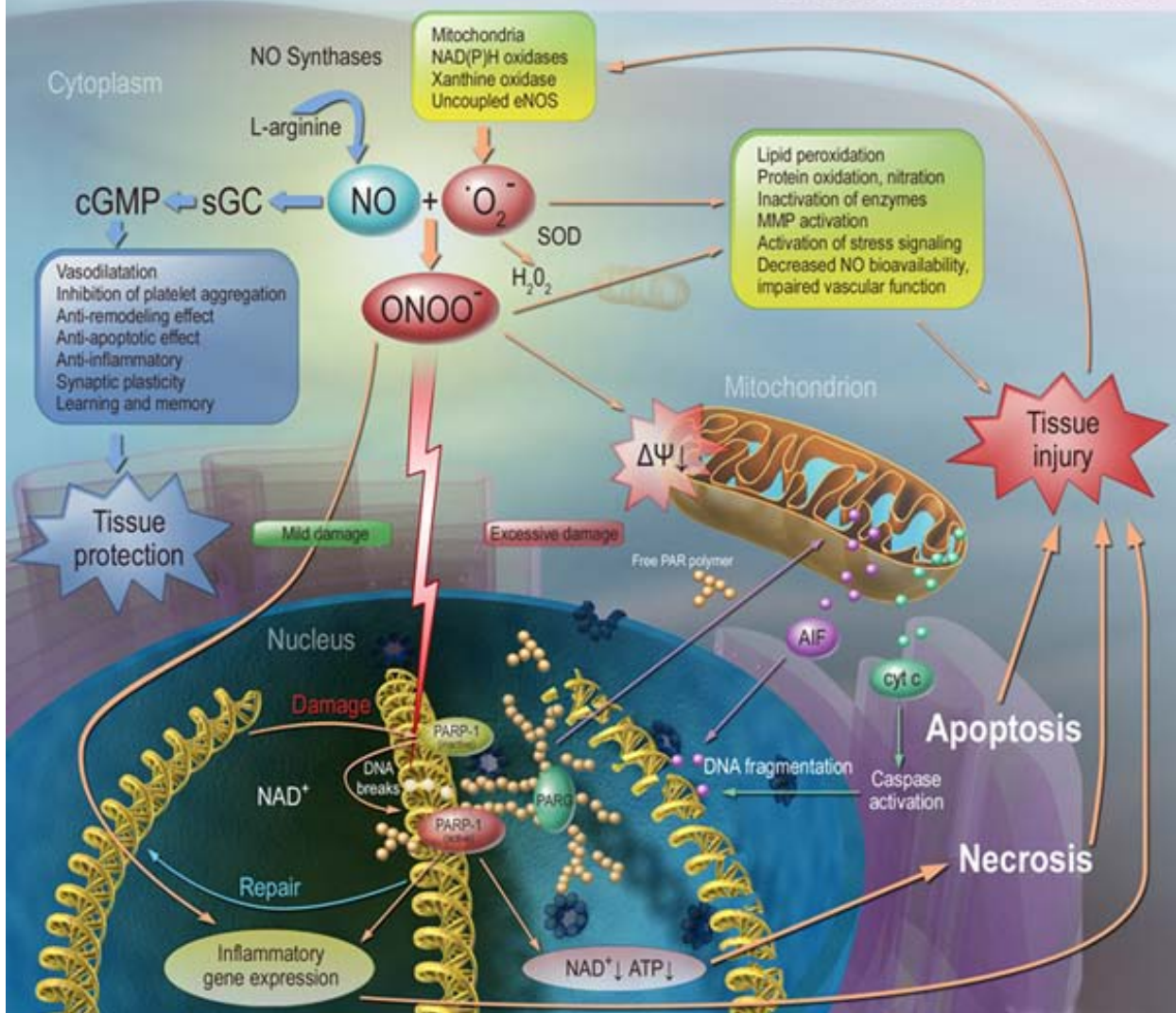


Fig. 1. Simplified scheme of the nitric oxide – peroxynitrite- PARP pathway in health and disease.

Nitric oxide (NO) by activating soluble guanylate cyclase (sGC)-cyclic guanosine-3',5'-monophosphate (cGMP) signal transduction pathway mediates various physiological/beneficial effects including synaptic plasticity, vasodilatation, inhibition of platelet aggregation, anti-inflammatory, anti-remodeling and anti-apoptotic effects, just mentioning a few. Under pathophysiological conditions (e.g. stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer and neurodegenerative disorders, etc.), nitric oxide and superoxide ($O_2^{\bullet -}$) react to form peroxynitrite ($ONOO^{\bullet}$)¹ which induces cell damage via lipid peroxidation, inactivation of enzymes and other proteins by oxidation and nitration and also via activation of stress signaling, matrix metalloproteinases (MMPs) among others. Mitochondrial enzymes are particularly vulnerable to attacks by peroxynitrite, leading to reduced ATP formation and induction of mitochondrial permeability transition by opening of the permeability transition pore (PTP), which dissipates the mitochondrial membrane potential ($\Delta\Psi$). These events result in cessation of electron transport and ATP formation, mitochondrial swelling and permeabilization of the outer mitochondrial membrane, allowing the efflux of several pro-apoptotic molecules, including cytochrome c and apoptosis-inducing factor (AIF). In turn, cytochrome c and AIF activate a series of downstream effectors which mediate caspase-dependent and independent apoptotic death pathways. In addition to its damaging effects on mitochondria, peroxynitrite, in concert with other oxidants, causes oxidative injury to DNA, resulting in DNA strand breakage which in turn activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP-1). Activated PARP-1 consumes NAD to build up poly(ADP-ribose) polymers (PAR) which are themselves rapidly metabolized by the activity of poly(ADP-ribose) glycohydrolase (PARG). Some free PAR may exit the nucleus and travel to the mitochondria, where they amplify the mitochondrial efflux of AIF (nuclear to mitochondria crosstalk). Depending on the severity of the initial damage by peroxynitrite and other oxidants, the injured cell may either recover or die. In the latter case, cell may be executed by apoptosis in case of moderate mitochondrial PTP opening and PARP-1 activation with preservation of cellular ATP, or by necrosis in case of widespread PTP opening and PARP-1 overactivation, leading to massive NAD consumption and collapse of cellular ATP. Overactivated PARP-1 also facilitates the expression of a variety of inflammatory genes leading to increased inflammation and associated tissue injury.

Our impending studies will also be directed towards the investigation of the role of oxidative/nitrosative stress related pathways in the development of complex hemodynamic alterations associated with diabetic cardiomyopathy, and the identification of novel therapeutic targets to counteract these pathological processes.

There is accumulating evidence indicating that endocannabinoids and synthetic cannabinergic ligands exert potent antioxidant, cytoprotective and antiinflammatory effects. Our recent studies showed that the non-psychoactive cannabinoid, cannabidiol, attenuated high glucose-induced endothelial cell activation and barrier disruption, which are crucial early events underlying the development of various diabetic complications and atherosclerosis.

Our future studies will also examine the role of the endocannabinoid system in the development of diabetic cardiovascular complications using mouse models of type 1 diabetes. These studies will also be extended to investigate the antioxidant/anti-inflammatory effects of various cannabinergic ligands on the development of oxidative stress and inflammation, and on cardiac and vascular dysfunction associated with advanced aging and doxorubicin-induced heart failure, conditions also known to be associated with increased oxidative/nitrosative stress and PARP activation, in relevant animal models.

In collaboration with Professor David Kass we have created, for the benefit of the cardiovascular research community, a comprehensive online resource tool for complex hemodynamic measurements in mice using a sophisticated pressure-volume (P-V) system. We have also used the P-V system in various collaborations to characterize complex cardiac function in interesting knockout mouse models of cardiovascular disorders (e.g., in collaboration with Drs. Usdin and Kunos). In the future we will develop novel multisegment catheters, which are expected to improve volume recordings in situations where the heart axis is abnormal (e.g., in various models of heart failure and myocardial hypertrophy).

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Role of the endocannabinoid system in tissue injury and inflammation.

During the last two years the main focus of our laboratory has been to understand the interplay of oxidative/nitrosative stress and inflammation with the endocannabinoid system (ES; an emerging very promising therapeutic target against various inflammatory and other diseases) in tissue injury associated with ischemia/reperfusion (I/R), doxorubicin-induced heart failure, and various *in vitro* and *in vivo* models of cardiovascular inflammation, as well as other pathologies associated with inflammation and tissue injury. These studies have demonstrated that oxidative/nitrosative stress is involved in the activation of the ES, and the stimulation of peripheral CB₂ cannabinoid receptors protected against I/R-induced tissue injury by decreasing endothelial cell activation and inflammatory response and interrelated oxidative/nitrosative stress (Fig. 2.).

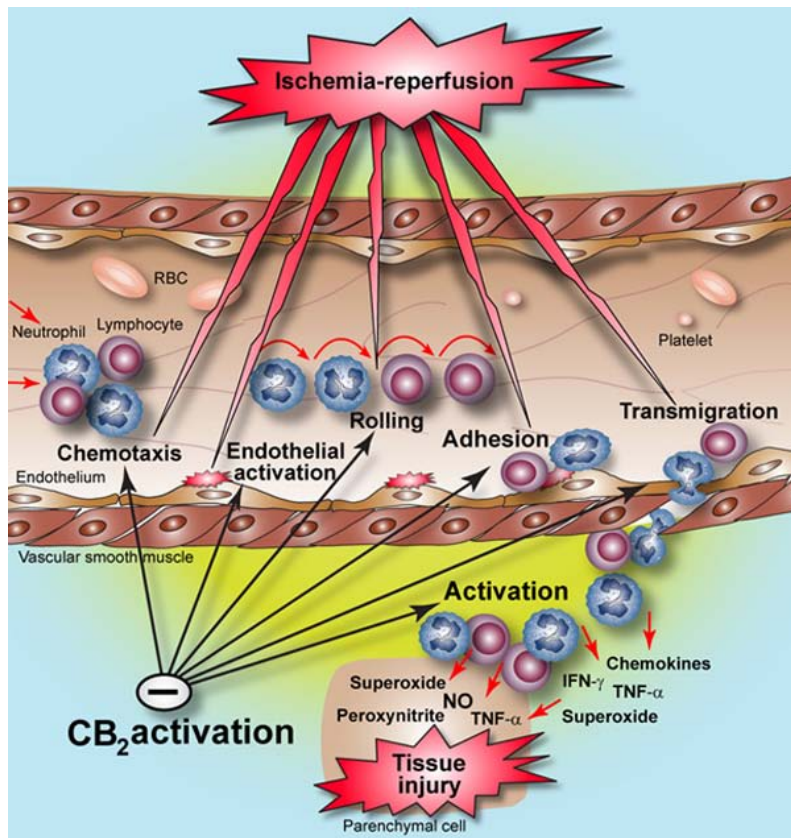


Fig. 2. Mechanisms of CB₂ receptor-dependent protection in ischaemia/reperfusion (I/R). CB₂ receptor agonists may protect against I/R injury by attenuating endothelial cell activation/inflammatory response, chemotaxis of inflammatory cells, rolling and adhesion of inflammatory cells to the endothelium, transendothelial migration, adhesion to the parenchymal cells and activation, and interrelated oxidative/nitrosative stress/inflammatory response.

Since the endothelial and smooth muscle cell activation and inflammatory response play a pivotal role in the development of atherosclerosis, we have also explored the effects of selective CB₂ receptor agonists (JWH133 and HU308) on these critical events using primary cultures of human coronary artery endothelial and smooth muscle cells (HCAEs and HCASMCs) and isolated vessels. We found that CB₂ agonists dose-dependently attenuated the TNF-• induced NF-•B and RhoA activation, up-regulation of adhesion molecules ICAM-1 and VCAM-1, increased expression of MCP-1, enhanced transendothelial migration of monocytes, and augmented monocyte-endothelial adhesion. Furthermore, the endotoxin-induced ICAM-1 and VCAM-1 expression in isolated aortas, and the adhesion of monocytes to aortic vascular endothelium were also decreased by CB₂ agonists. TNF-• triggered proliferation and migration of HCASMCs and activation of various interrelated signaling pathways (Ras, p38 MAPK, ERK 1/2, SAPK/JNK and Akt), which could be dose-dependently attenuated by CB₂ agonists.

Our studies have also demonstrated marked protection of CB₁ antagonists against Doxorubicin-induced cell death in cardiomyocytes and associated heart failure.

Our impending studies will also be directed towards the understanding of the mechanisms of the activation of the endocannabinoid system during reperfusion injury and on the elucidation of the role of endocannabinoid system in various models of cardiomyopathy and heart failure (e.g. doxorubicin-induced heart failure and diabetic cardiomyopathy) particularly focusing on the signaling mechanisms involved in these effects.

Our recent collaborative studies with Dr. Rohini Kuner have established an important role for the endocannabinoid system in acute and chronic pancreatitis and pain.

The above mentioned studies may identify new pharmacological targets in various forms of tissue injury and cardiovascular dysfunction associated with increased inflammation and tissue injury.

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Role of oxidative-nitrosative stress in ethanol-induced tissue-damage.

Moderate and heavy drinking may significantly influence cardiovascular function and aging in different ways. During the course of the last decade, several research groups have reported that, in animal models of myocardial ischemia/reperfusion, ethanol and non-ethanol components of wine may have a specific protective effect on the myocardium, independent of the classical risk factors implicated in vascular atherosclerosis and thrombosis. Apoptosis is a mechanism of cell death implicated in the pathogenesis of alcohol-induced organ damage. Experimental studies have suggested alcohol-mediated apoptosis in cardiac muscle, and there is also evidence of skeletal muscle apoptosis in long-term high-dose alcohol drinkers. Apoptosis is present to a similar degree in the heart muscle of high-dose alcohol drinkers and long-standing hypertensive subjects and is related to structural damage.

We have recently developed an assay allowing simultaneous quantitative detection of oxidative stress and apoptosis in virtually any live cells, which we utilized in our studies and in various collaborations. Our future studies will be focused on the understanding of the mechanisms of ethanol-induced oxidative/nitrosative stress and apoptosis in the cardiovascular system and also in other organ systems. We will use clinically relevant models of aging (Fisher rats developed by

the National Institute on Aging) to address the effects of ethanol on the course of oxidative/nitrosative stress and inflammation associated with aging.

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