Endocannabinoids and Liver Disease.

II. Endocannabinoids in the pathogenesis and treatment of liver fibrosis

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Siegmund SV, Schwabe RF. Endocannabinoids and liver disease. II. Endocannabinoids in the pathogenesis and treatment of liver fibrosis. Am J Physiol Gastrointest Liver Physiol 294: G357–G362, 2008. First published November 15, 2007; doi:10.1152/ajpgi.00456.2007.—Hepatic fibrosis is the response of the liver to chronic injury and is associated with portal hypertension, progression to hepatic cirrhosis, liver failure, and high incidence of hepatocellular carcinoma. On a molecular level, a large number of signaling pathways have been shown to contribute to the activation of fibrogenic cell types and the subsequent accumulation of extracellular matrix in the liver. Recent evidence suggests that the endocannabinoid system is an important part of this complex signaling network. In the injured liver, the endocannabinoid system is upregulated both at the level of endocannabinoids and at the endocannabinoid receptors CB1 and CB2. The hepatic endocannabinoid system mediates both pro- and antifibrogenic effects by activating distinct signaling pathways that differentially affect proliferation and death of fibrogenic cell types. Here we will summarize current findings on the role of the hepatic endocannabinoid system in liver fibrosis and discuss emerging options for its therapeutic exploitation.

Pathophysiology of Hepatic Fibrogenesis

Liver fibrosis is the common wound-healing response to chronic hepatic injury of various etiologies including alcohol abuse, viral and parasitic infection, nonalcoholic steatohepatitis, drugs, iron and copper overload, and autoimmune diseases (2). Acute liver injury leads to a limited wound-healing response and a temporary increase in a collagen-rich extracellular matrix (ECM), which provides a scaffold for the restoration of liver architecture and function. In contrast, chronic liver injury is associated with a chronic and exaggerated deposition of ECM that undergoes additional changes that render it more resistant to degradation. In advanced stages of injury, hepatocytes are replaced by abundant ECM leading to decreased liver function as well as increased vascular resistance and portal hypertension. Hepatic stellate cells (HSCs, also known as Ito cells, fat storing cells, or hepatic lipocytes) have been shown to be a predominant source of ECM in the fibrotic liver (2). In the normal liver, HSCs produce only negligible amounts of ECM and their main function is the storage of large amounts of vitamin A. Following liver injury, HSCs activate and differentiate into myofibroblast-like cells that express large amounts of ECM proteins, acquire contractile properties, and secrete proinflammatory mediators (2). In recent years, it has been suggested that ECM-producing myofibroblasts can also derive from other sources, including portal fibroblasts and bone marrow. Moreover, it has been proposed that hepatocytes undergo epithelial-mesenchymal transition and contribute to the pool of myofibroblasts in the injured liver. Although it is currently accepted that HSCs are not the only source of ECM in the fibrotic liver, the contribution of portal fibroblasts, bone marrow, and hepatocytes to ECM synthesis needs to be further characterized in different types of liver injury.

Removal of the causative agent is the most effective therapy for treating hepatic fibrosis (2). Following eradication of hepatitis C virus infection, cessation of alcohol abuse, treatment of hemochromatosis, or resolution of biliary obstruction, liver fibrosis can significantly reverse and sometimes even result in a completely normal hepatic architecture (2). Fibrosis resolution is preceded by the disappearance of activated HSCs, suggesting that endogenous mediators in the liver either reverse HSC activation or induce cell death in HSCs. There is a

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growing body of evidence that HSCs undergo apoptosis during the resolution of hepatic fibrosis and that the selective induction of HSC cell death may be a tool to reduce fibrogenesis under circumstances in which the underlying disease cannot be cured (12). Endocannabinoids are among the most potent endogenous inducers of HSC death in vitro, suggesting that they may be involved in limiting hepatic fibrogenesis in vivo. Currently, there is no effective treatment for hepatic fibrosis except treating the underlying disease. New concepts for the treatment of hepatic fibrosis focus on pathways that mediate HSC activation and proliferation as well as mediators that induce cell death of HSCs. Therefore, endocannabinoids have received much attention as potential antifibrogenic mediators.

Endocannabinoids and Endocannabinoid-Degrading Enzymes in the Normal and Fibrotic Liver

The normal liver contains only low levels of the endocannabinoids AEA and 2-AG (13, 18, 21, 23, 25). This is, at least in part, the result of high hepatocellular expression of fatty acid amide hydrolase (FAAH), the most important enzyme for AEA degradation (6). Activation of the hepatic endocannabinoid system can be detected already in early stages of liver disease. Hepatic and serum levels of AEA increase within days of liver disease such as fatty liver and acute hepatitis (5, 18). In fatty liver, elevation was not caused by an upregulation of the synthesis pathway of AEA, but by a decrease in AEA degradation by FAAH (see the planned future article by Kunos et al. in this Themes series). In the early stages of cholestatic liver disease, FAAH mRNA and FAAH activity are also decreased (23), supporting the hypothesis that changes in endocannabinoid degradation are an important mechanism to control endocannabinoid levels in the liver. The levels of 2-AG are also significantly upregulated in acute liver injury induced by bile ligation or injection of a single dose of CCL_4 (22). However, it remains to be investigated whether a decline in the expression of monoacylglycerol lipase, the primary enzyme for 2-AG degradation (10), contributes to the increase of 2-AG levels in the acutely injured liver. In the later stages of liver fibrosis, hepatic or serum levels of endocannabinoids remain significantly increased. Although data on endocannabinoid levels in early liver injury are limited to rodents, it has been shown that endocannabinoids are elevated in patients with advanced liver disease. Patients with liver cirrhosis display high serum levels of AEA (5) as well as increased levels of AEA in circulating monocytes (3, 19). Lipopolysaccharide (LPS) induces AEA levels in macrophages and this process is, at least in part, dependent on NF-κB (14). The LPS-induced increase in AEA levels in macrophages is mediated by de novo synthesis and not by reduced degradation, since LPS fails to decrease FAAH expression and activity (14). The synthesis of 2-AG in macrophages can also be triggered by LPS, but to a much lesser extent. The well-documented elevations of LPS in hepatic fibrosis (20) suggest that LPS is a key trigger of endocannabinoid synthesis in the fibrotic liver. A role of other mediators and pathways in hepatic endocannabinoid synthesis is likely but has not yet been determined. Moreover, there are no data on the contribution of other cell types, such as hepatocytes or HSCs, to endocannabinoid synthesis in early and late stages of liver disease. However, it is well known that hepatocytes express high levels of FAAH (23). In mice, hepatic FAAH expression and activity are the highest in the body and even exceed those of the brain. This implies a crucial role for hepatocytes in limiting hepatic endocannabinoid levels. Additionally, high levels of FAAH render hepatocytes resistant to endocannabinoid-induced signals (23). In contrast to hepatocytes, HSCs do not express significant amounts of FAAH and are therefore more sensitive to AEA. There are no significant differences in MGL expression between hepatocytes and HSCs (22).

Endocannabinoid Receptors in the Normal and Fibrotic Liver

The expression of endocannabinoid receptors is nearly undetectable in the normal liver, providing further evidence to the notion that the endocannabinoid system is in a low activation state in the normal liver (13, 18, 25). Both CB1 and CB2 receptors are upregulated in the early stages of liver injury. Expression levels remain elevated in the late stages of liver injury, but strong upregulation of CB1 occurs only in some types of liver injury whereas CB2 is strongly upregulated in all types of liver injury (4, 13, 18, 21, 25). CB1 receptors are significantly upregulated in the vascular endothelium as well as in myofibroblasts located in fibrotic bands of cirrhotic livers in humans and rodents (25). CB1 receptors are also expressed in hepatocytes of human and rodent fatty livers (see the planned future article by Kunos et al. in this Themes series), but it remains unknown whether other types of liver injury upregulate CB1 receptors on hepatocytes. In vitro studies reveal that quiescent HSCs do not display a notable expression of CB1 receptors, and that CB1 expression is only induced after culture activation (13, 24, 25). In CCl_4-treated mouse livers, CB2 receptors are also expressed in myofibroblasts as well as in inflammatory cells and biliary epithelial cells. In patients with hepatic cirrhosis, CB2 receptors are expressed in myofibroblasts. Moreover, CB2 expression is upregulated in cultured myofibroblasts isolated from human liver and is upregulated during HSC culture activation (13). However, a recent microarray analysis failed to show a significant upregulation of CB1 and CB2 in vivo-activated HSCs isolated from mice that underwent bile duct ligation or CCl_4 injections (7). In summary, the induction of the endocannabinoid system at both the level of endocannabinoids and their receptors constitutes two complementary mechanisms that render the liver responsive toward endocannabinoids in the course of hepatic fibrogenesis.

Role of the Endocannabinoid System in Hepatic Fibrogenesis

The crucial role of the endocannabinoid system in hepatic fibrosis has been highlighted by recent studies in which CB1 and CB2 receptors were blocked by both genetic and pharmacological approaches. Interestingly, CB1 and CB2 exert opposing effects on fibrogenesis, suggesting that the endocannabinoid system regulates both pro- and antifibrogenic responses in the liver (see Fig. 1). CB1-deficient mice show a strong decrease in fibrogenesis induced by CCL_4, thioacetamide, or bile duct ligation (25), whereas CB2-deficient mice display an increase in fibrogenesis following CCL_4 treatment (13). The profibrogenic function of the CB1 receptor in the liver is further emphasized by the ability of the CB1 inhibitor rimonabant to reduce profibrogenic markers such as TGF-β1 and α-smooth muscle actin as well as histological fibrosis after bile duct...
ligation or treatment with thioacetamide or CCl₄ (25). It was suggested that an increase in cell death and a reduced proliferation of myofibroblasts are responsible for the observed decrease in fibrosis after CB1 antagonism. The effects of CB2 antagonism appear to be exactly opposite of CB1 antagonism, namely decreased myofibroblast cell death and enhanced proliferation (13). Interestingly, patients with chronic hepatitis C and daily marijuana consumption displayed more severe fibrosis progression than nonconsumers, suggesting that profibrogenic CB1 signals dominate over antifibrogenic CB2 signals (11). However, one has to keep in mind that CB2 is predominantly expressed in monocytic cell types and that it exerts immunosuppressive effects that may have confounding effects in patients with chronic hepatitis C because these patients require an intact immune response to keep the hepatitis C virus in check. It is conceivable that chronic marijuana consumption promotes fibrogenesis in chronic hepatitis C via CB2-mediated suppression of antiviral immunity (21).

A main weakness of current study designs is their inability to pinpoint cellular targets of endocannabinoids in vivo. Although the above-described studies postulate that the pro- and antifibrogenic effects of CB1 and CB2 receptors are predominantly mediated through hepatic myofibroblasts, it is possible that other cell types make major contributions, since it has been shown that CB1 and CB2 are highly expressed in other hepatic cell types. In the injured liver, CB1 is expressed on the vascular endothelium and in hepatocytes and may thus alter the response to injury in these cell types. Because CB2 is predominantly expressed in monocytic cell types and exerts strong anti-inflammatory effects, CB2 may mediate its antifibrotic actions through anti-inflammatory signals in patients with nonviral causes of hepatic fibrosis. Thus investigations in isolated hepatic cell populations as well as in mouse models with cell-specific CB1 and CB2 deletions are required to further understand the targets of endocannabinoids and to determine how CB1- and CB2-mediated signals modulate fibrogenesis. Also, the liver is innervated with axonal processes of autonomic nerve fibers being in close contact with HSCs, raising the possibility that cannabinoid receptors could influence this interaction, and thereby HSC activation and fibrogenesis.

Effects of Endocannabinoids on Hepatic Cell Populations

Although the above-mentioned in vivo studies show an important role of the endocannabinoid system in hepatic fibrogenesis, the molecular signals that link endocannabinoids to fibrosis are not fully understood. Endocannabinoids are known to exert numerous and sometimes opposite effects on target cells. Recent in vitro observations of how endocannabinoids affect fibrogenic cell types are the basis for pathophysiological concepts on the role of endocannabinoids during fibrogenesis in vivo. Currently, it is believed that the regulation of cell death and proliferation in fibrogenic cell types are the two main mechanisms by which endocannabinoids influence hepatic fibrogenesis (see Fig. 1). The modulation of immune responses comprises a third potential mechanism by which endocannabinoids modify wound-healing responses in the liver.

Endocannabinoid-Induced Cell Death

There is a growing body of evidence that endocannabinoids control cell death and survival both in vitro and in vivo (15). In many cell types, endocannabinoids induce apoptotic cell death at supraphysiological concentrations in the micromolar range.
Endocannabinoids mediate apoptosis not only through CB1 and CB2, but also through transient receptor potential vanilloid 1 (TRPV1), which also acts as receptor for anandamide, and in a receptor-independent manner. The role of AEA in cell death regulation is well studied whereas there is little data on cell death induction by other endocannabinoids. Several mechanisms of AEA-induced cell death have been described: 1) Stimulation of CB1 and CB2 receptors induces the activation of factor associated with neutral sphingomyelinase, which leads to short-term increases in ceramide levels, and an additional activation of the de novo pathway of ceramide synthesis, which induces a sustained generation of ceramide and apoptosis. 2) AEA leads to activation of an ASK-JNK-p38 cascade followed by sustained activation of JNK and cell death. 3) Activation of TRPV1 triggers a rise in cytosolic Ca\(^{2+}\), cyclooxygenase-2, and lipoxygenase activation, a drop in mitochondrial membrane potential, cytochrome c release, and caspase 3 activation. 4) Endocannabinoids induce receptor-independent cell death that is mediated by an increase in reactive oxygen species (ROS) generation and yet uncharacterized downstream signals that subsequently lead to caspase-3-independent necrosis. 2-AG induces cell death through mitochondrial ROS generation and caspase 3 activation in a cannabinoid (CB) receptor-independent manner (13, 22). It should be emphasized that in vivo concentrations of 2-AG are ∼100-fold higher than those of AEA and that 2-AG is thus more likely to reach the threshold for cell death induction in vivo. In some cell types including neuronal cells, CB1 or CB2 receptors prevent apoptosis by activating cytoprotective pathways such as phosphatidylinositol 3-kinase through CB1. This is of interest because HSCs share many features and molecular markers with neuronal cells.

HSCs are highly sensitive to cell death induction by exo- and endocannabinoids (13, 22, 24). Cell death induction by endocannabinoids such as AEA and 2-AG requires the presence of membrane cholesterol, but not CB1, CB2, or TRPV1 (13, 22, 24), whereas cell death induced by exocannabinoids requires CB2 (13). Endocannabinoids induce a rapid increase of mitochondrial ROS production which is responsible for the induction of cell death as shown by the ability of various antioxidants to block 2-AG- and AEA-mediated cell death in HSCs (see Fig. 2). Although the upstream requirements for AEA- and 2-AG-induced cell death are highly similar, there are significant differences in downstream signals and the mode of cell death. Whereas AEA-treatment ultimately leads to necrosis, the majority of 2-AG-treated HSCs display features of apoptosis such as poly(ADP-ribose) polymerase and caspase 3 cleavage and positive annexin V staining. It is conceivable that AEA and 2-AG activate similar pathways in HSCs and that AEA-induced cell death is an aborted form of apoptosis that rapidly converts into necrosis. Accordingly, some AEA-treated HSCs display annexin V staining at very early time points after treatment (R. F. Schwabe, unpublished observation). In contrast to HSCs, primary hepatocytes are completely resistant to cell death induced by AEA or 2-AG, even at extremely high doses, suggesting the possibility of selective cell death induction by endocannabinoids (22, 24). High expression and activity of endocannabinoid-degrading enzymes (e.g., FAAH), high levels of antioxidants such as glutathione, and lower membrane cholesterol levels are the main factors that render hepatocytes resistant to endocannabinoid-induced cell death (23, 24). Indeed, hepatocytes are sensitized to endocannabinoids when FAAH activity is blocked or glutathione is depleted (23, 24). Exocannabinoids have been shown to mediate myofibroblast cell death through a CB2-dependent mechanism (see Fig. 1). In hepatic myofibroblasts, CB1 appears to mediate cytoprotective signals and protects against spontaneous agonist-independent cell death even in the absence of endocannabinoid ligands (25). These results raise the possibility that CB1 possesses signaling capacity even in the absence of ligand activation. Accordingly, CB1-deficient myofibroblasts show a lower baseline activation of ERK and Akt, two kinases which mediate proproliferative and antiapoptotic effects in myofibroblasts and HSCs (25). Further studies are required confirm the importance of the postulated ligand-independent CB1 pathway in HSCs and myofibroblasts. Since HSC cell death reduces hepatic fibrogenesis, it is conceivable that selective cell death induction in HSCs by high concentrations of exo- or endocannabinoids may reduce hepatic fibrogenesis whereas the activation of cytoprotective pathways by CB1 increases fibrogenesis. It remains to be determined, however, whether endocannabinoids can reach sufficient levels to induce HSC death in vivo and whether CB2-dependent and receptor-independent proapoptotic signals can overcome CB1-dependent protective signals. Daily consumption of marijuana does not decrease but rather increases the progression of hepatic fibrosis, suggesting a dominance of CB1-dependent profibrogenic.

Fig. 2. Model of endocannabinoid-induced death pathway in hepatic stellate cells. AEA or 2-AG binds to cholesterol-rich lipid rafts in the cell membrane, followed by mitochondrial generation of cytotoxic reactive oxygen species (ROS). In 2-AG-treated cells, mitochondria subsequently release cytochrome c that activates caspase 3, which can be inhibited by z-VAD-fmk (z-VAD). AEA-treated cells undergo necrosis. Inhibition of endocannabinoid-induced cell death can be achieved by β-MCD (disruption of lipid rafts), antioxidants (scavenging mitochondrial ROS) but not by pharmacological or genetic ablation of CB1 or CB2 signaling. FAAH protects from cell death by degrading AEA, and FAAH overexpression can block AEA-induced ROS generation and cell death.
pathways (11). Inhibiting endocannabinoid-degrading enzymes such as FAAH and MGL represents an effective strategy to increase endocannabinoid levels in vivo and to potentially induce HSC and myofibroblast death. However, FAAH protects hepatocytes from AEA-induced cell death and inhibition of FAAH may thus adversely affect the injured liver (23).

**Endocannabinoids and Myofibroblast Proliferation**

Proliferation of HSCs and myofibroblasts is a crucial feature of hepatic fibrogenesis and increases the pool of activated myofibroblasts in the liver. Endocannabinoids including 2-AG and AEA exert antiproliferative actions in a wide spectrum of cell lines as well as primary cells. Several pathways are believed to contribute to endocannabinoid-induced growth arrest, such as 1) inhibition of p21ras, and the ras cascade-dependent proliferation in tumors; 2) prolonged activation of ERK, which does not induce, but instead inhibits proliferation; and 3) inhibition of growth factor signaling. However, in some cell types, such as lymphocytes and neuronal cells, AEA as well as 2-AG can also induce proliferation in certain stages of cell development by a CB receptor-mediated mechanism that is preceded by ERK activation.

We and others have shown that nontoxic concentrations of AEA or 2-AG significantly reduce HSC proliferation in the presence of PDGF (13, 24). Endocannabinoids reduce myofibroblast proliferation in a receptor-independent manner, whereas exocannabinoids such as THC decrease myofibroblast proliferation in a CB2-dependent manner (13, 24). Similar to neuronal cells, myofibroblasts appear to proliferate in response to CB1 activation. Although a direct proproliferative effect of endocannabinoids has not been demonstrated in hepatic myofibroblasts, CB1-deficient myofibroblasts displayed a strong decrease in spontaneous proliferation in the absence of endocannabinoids that was postulated to be mediated by the same ligand-independent CB1 signals that mediate antiproliferative signals in myofibroblasts (25). CB1-deficient myofibroblasts displayed a decrease in ERK and Akt phosphorylation, two kinases that are crucial for proliferation in hepatic myofibroblasts and HSCs (25). One should keep in mind that in vivo concentrations of endocannabinoids are lower than the concentrations that were associated with antiproliferative effects in vitro. Thus it is possible that ligand-independent CB1-mediated proproliferative effects dominate over CB2-dependent and CB receptor-independent antiproliferative effects during fibrogenesis.

**Anti-Inflammatory Effects of Endocannabinoids**

Hepatic inflammation is tightly linked to hepatic fibrosis in experimental models of fibrogenesis and in patients with chronic liver injury. Kupffer cells are crucial for hepatic fibrogenesis and directly promote the activation of HSCs. Other cells of the immune system such as activated T lymphocytes and neutrophils are believed to additionally contribute to the inflammatory microenvironment of the injured liver that drives the perpetuation of HSC activation and fibrogenesis. Kupffer cells express high amounts of CB2 mRNA (R. F. Schwabe, unpublished observations). CB2 exerts strong anti-inflammatory effects, suggesting a potential role of endocannabinoids in the regulation of hepatic inflammation and Kupffer cell activation in a CB2-dependent manner. CB2 mediates anti-inflammatory effects through several mechanisms which include the suppression of proinflammatory cytokines such as TNF-α and IFN-γ, the stimulation of anti-inflammatory cytokines such as IL-10, and inhibition of leukocyte rolling and subsequent macrophage migration to the site of injury (see the planned future article by Gao and Pacher in this Themes series). Thus CB2-mediated anti-inflammatory effects might account for some of antifibrogenic effects of endocannabinoids in the liver.

**Effects of the Endocannabinoid System on Portal Hypertension and Other Complications of Hepatic Fibrosis and Cirrhosis**

Portal hypertension with the subsequent development of esophageal varices and variceal bleeding is one of the most dangerous complications of liver fibrosis. Portal hypertension is, at least in part, caused by mesenteric vasoconstriction and the associated peripheral vasodilatation. Interestingly, CB1 receptors are expressed in vascular endothelial cells and contribute to systemic hypotension and portal hypertension in advanced liver cirrhosis (3, 19) (see the planned future article by Lee and Gaskari in this Themes series). The blockade of CB1 receptors by rimonabant increases mean arterial pressure and peripheral resistance and reduces mesenteric blood flow in rats with CCl4-induced cirrhosis (3, 19). Because peripheral vasodilatation and mesenteric vasoconstriction are intimately linked to the pathophysiology of ascites formation, it is possible that CB1 antagonism will have beneficial effects not only on portal hypertension, but also on ascites formation. Hepatic encephalopathy is another complication of end-stage liver disease, and application of the CB1 antagonist rimonabant significantly alleviates symptoms of hepatic encephalopathy in thioacetamide-treated cirrhotic rats (1).
yet-unidentified endocannabinoid mediates CB1-dependent profibrogenic effects or whether CB1 receptors generate pro-fibrogenic signals in a ligand-independent manner. Moreover, further studies are needed to investigate the potential role of CB2-dependent anti-inflammatory effects in fibrogenesis and the ability and relevance of endocannabinoid-mediated cell death of fibrogenic cells in vivo. The generation of mice with cell-specific deletion of components of the endocannabinoid system will answer some of these questions in the future. Most importantly, it needs to be determined whether CB1 antagonists is a feasible strategy in patients with fibrosis because the concentrations of the CB1 antagonist rimonabant that were required to block fibrogenesis in mice were 20-fold higher than those used in overweight patients. Thus chances of side effects of CB1 antagonists such as depression may be even higher, and results of current investigations on the safety of CB1 antagonists should be awaited before their use in patients with fibrosis should be considered.

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