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Original Paper

Ultra Low Dose Delta 9-Tetrahydrocannabinol Protects Mouse Liver from Ischemia Reperfusion Injury

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Key Words

Liver • Ischemia/reperfusion • Delta 9-tetrahydrocannabinol • Ultralow dose

Abstract

Background/Aims: Ischemia/reperfusion (I/R) injury is the main cause of both primary graft dysfunction and primary non-function of liver allografts. Cannabinoids has been reported to attenuate myocardial, cerebral and hepatic I/R oxidative injury. Delta-9-tetrahydrocannabinol (THC), a cannabinoid agonist, is the active components of marijuana. In this study we examined the role of ultralow dose THC (0.002mg/kg) in the protection of livers from I/R injury. This extremely low dose of THC was previously found by us to protect the mice brain and heart from a variety of insults. **Methods:** C57Bl Mice were studied in *in vivo* model of hepatic segmental (70%) ischemia for 60min followed by reperfusion for 6 hours. **Results:** THC administration 2h prior to the induction of hepatic I/R was associated with significant attenuated elevations of: serum liver transaminases ALT and AST, the hepatic oxidative stress (activation of the intracellular signaling CREB pathway), the acute proinflammatory response (TNF-α, IL-1α, IL-10 and c-FOS hepatic mRNA levels, and ERK signaling pathway activation). This was followed by cell death (the cleavage of the pro-apoptotic caspase 3, DNA fragmentation and TUNEL) after 6 hours of reperfusion. Significantly less hepatic injury was detected in the THC treated I/R mice and fewer apoptotic hepatocytes cells were identified by morphological criteria compared with untreated mice. **Conclusion:** A single ultralow dose THC can reduce the apoptotic, oxidative and inflammatory injury induced by hepatic I/R injury. THC may serve as a potential target for therapeutic intervention in hepatic I/R injury during liver transplantation, liver resection and trauma.

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Introduction

Ischemia–reperfusion (I/R) injury is the main cause of both primary graft dysfunction (occurring in 10–30 % of grafts) and primary non-function of liver allograft (occurring in 5 % of grafts) [1]. The latter is responsible for 81 % of re-transplantation during the first week after surgery [1, 2]. Therefore, minimizing the adverse effects of I/R injury could increase the number of both suitable transplantation grafts and patients who successfully recover from liver transplantation. Cellular and biochemical processes occurring during hepatic I/R are diverse and complex and include cellular immune activation, cytoprotective functions and immune regulation. Nevertheless, a significant part of these processes are still unknown or unclear. The deleterious effects of I/R arise from the acute generation of reactive oxygen (ROS) subsequent to re-oxygenation upon vascular re-opening. These compounds cause direct tissue damage and initiate a chain of deleterious cellular responses leading to inflammation and cell death, and eventually to target organ failure [3].

Cannabinoids and their analogues, exert their effects by activating at least two specific receptors (CB1 and CB2) that belong to the seven transmembrane G-protein coupled receptor (GPCR) family. Both CB1 and CB2 cannabinoid receptors mRNA and proteins are present in the liver [4-6]. However, basal hepatic expression of cannabinoid receptors is faint, with low levels of CB2 receptors in Kupffer cells and of CB1 receptors in endothelial cells and hepatocytes. Thus, CB2 receptors may undergo significant up-regulation in Kupffer cells and hepatic myofibroblasts, whereas CB1 receptors are induced in hepatocytes, hepatic myofibroblasts and endothelial cells [7]. Oxidative stress and inflammatory stimuli may trigger hepatic endocannabinoid production [6]. The protective effect of various cannabinoid agonists has previously been demonstrated in a well characterized mouse model of hepatic I/R injury [6-12]. All these cannabinoids has been applied at high doses (3-20 mg/kg) that also induce the conventional pharmacological effects of cannabinoids.

We have recently shown that a single i.p. administration of Δ9- tetrahydrocannabiol (THC), the major psychoactive ingredient of marijuana, at an extremely low dose (0.002 mg/kg, which is 3–4orders of magnitude lower than doses that evoke the conventional acute effects of the drug), protected the mice and prevented the development of long-term cognitive deficits induced by the epileptogenic drug pentylenetetrazole , by pentobarbital deep anesthesia or by CO intoxication [10-12]. In addition we have demonstrated that a single ultra low dose of THC before ischemia is a safe and effective treatment that reduces myocardial ischemic damage [13]. The purpose of the present study was to test and characterize hepatoprotective effects of a very low dose (0.002 mg/kg) of THC, a dose that does not induce any of psychotropic and somatic acute effects of cannabinoid drugs, administered 2 h before I/R in mice *in vivo*. The results obtained demonstrate that THC is hepatoprotective as seen by various functional, biochemical and histological markers of liver damage.

Materials and Methods

All male mice were maintained in a pathogen-free facility and were fed pellet food and water ad libitum, until the start of the experiment (at 12 weeks old). All experiments were carried out in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

General experimental protocols

In vivo I/R injury model. A model of segmental (70 %) hepatic ischemia was used as previously described [14]. Adult C57BL male mice weighing 25–28 g were anesthetized by intra-peritoneal injection of Ketamine (100 mg/kg) and Xylazine (10mg/kg). After a midline laparotomy, all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were occluded for 60 min with a vascular a traumatic clamp followed by 6 hours of reperfusion. This method of partial hepatic ischemia

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prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. Reperfusion was initiated by removal of the clamp. THC (donated by NIDA, USA) was injected i.p. in a vehicle solution consisting of 1:1:18 of ethanol:cremophor (Sigma–Aldrich):saline. Mice were randomly allocated to four main experimental groups (n = 10 each): (1) Control group undergoing sham operation. (2) Similar to group 1 with the administration of THC 2 h before the operation, or administrating vehicle and not THC. (3) Mice undergoing *in vivo* hepatic I/R. (4). Similar to group 3 with the administration of THC 2 h before the induction of ischemia.

Liver enzyme levels. Serum (0.5 ml) was collected at 6 h post reperfusion and kept on ice until processed. Levels of aspartate transaminase (AST) and alanine transaminase (ALT) were determined in serum in duplicate, using commercial kits according to the manufacturer's protocols.

Pathological evaluation

Specimens from the ischemic liver in all groups were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin. Pathological findings were assessed by one of the author (OP) blinded to the group allocations. Apoptotic cells were identified by morphological criteria (cell shrinkage, chromatin condensation, margination, apoptotic bodies).

DAPI staining and TUNEL Assay

Apoptotic cells were identified using the DeadEnd™ flourometric TUNEL system (Promega) according to the manufactured instruction followed by nuclear staining with DAPI (Life Technologies).

Western blot analysis of liver tissue

Liver tissue samples were homogenized in RIPA lysis buffer and quantified for protein levels using a commercial assay (Bio-Rad). Liver extracts (40 µg protein/ lane) were electrophoresed and subjected to SDS-PAGE under reducing conditions using 12.5 % polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with the appropriate antibodies. (ERK, phospho-ERK, CREB, phospho-CREB, Caspase-3). Antibodies against ERK were purchased from sigma and used in 1:10000 dilutions. Antibodies against p-ERK CREB, p-CREB and Caspase-3 were purchased from cell signaling and used in 1:1000 dilution.

RNA purification and real-time qPCR analysis

Total RNA was extracted from liver samples using TRI-Reagent (Sigma), followed by treatment with 1U of RNase-free DNase (Roche). Reverse transcription was performed on 2 µg total RNA using High Capacity cDNA. Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. qPCR was performed using SYBR Green Real-Time PCR Kit (Applied Biosystems) according to manufacturer's recommendations with gene specific primers for IL1α, TNFα, IL10 and cFOS (listed in Table 1) in an ABI Step ONE Plus system (Applied Biosystems). HPRT and βActin were used as a reference endogenous control. All the reactions were performed in duplicates and relative gene expression values determined using the $2^{-\Delta\Delta Ct}$ method with ABI Prism 7000 SDS (Applied Biosystems).

Statistical analysis

Results are expressed as mean ± standard error. Differences between groups were assessed using the analysis of variance the 2 way ANOVA following log transformation to approach normal distribution.

Table 1. Sequences of primers used for quantitative real-time PCR

	Forward	Reverse
mIL-1α	GAGTCGGCAAAGAAATCAAGATG	CAATGGCAGAACTGTAGTCTTCGT
mTNFα	GCCACCACGCTCTTCTGTCTAC	GGGTCTGGGCCATAGAACTGAT
m IL10	GGCGCTGTCATCGATTTCTC	CACCTTGGTCTTGGAGCTTATTAAA
mcFOS	TCCAAGCGGAGACAGATCAAC	TTTTTCCTTCTCTTTCAGCAGATTG
mHPRT	AGCTACTGTAATGATCAGTCAACG	AGAGGTCCTTTTCACCAGCA
mβactin	CCTGTATGCCTCTGGTCGTA	CCATCTCCTGCTCGAAGTCT

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Results

Reduced liver enzymes level following the administration of THC

Liver enzyme levels remained within the normal range in the control, sham operated group for the duration of the study. Moreover, the administration of THC to sham operated mice did not affect significantly the mean serum levels of ALT/AST. A statistically significant difference was noted in mean serum ALT/AST levels following the induction of I/R, compared with sham operated mice ($p=0.0199$, $p=0.0169$), respectively. In THC pretreated mice following the induction of I/R, the increase in serum liver enzymes level was significantly less compared with untreated mice ($p = 0.022$, $p = 0.026$), respectively (Fig. 1). A statistically significant difference was noted in mean serum ALT/AST levels between the two untreated and I/R THC treated mice ($p=0.04$ and 0.05, respectively) (Fig.1). Significant differences were noted in liver enzymes level between the different variants (ALT IR p=<0.001 THC p=0.029 AST IR p=0.001 THC p=0.024)
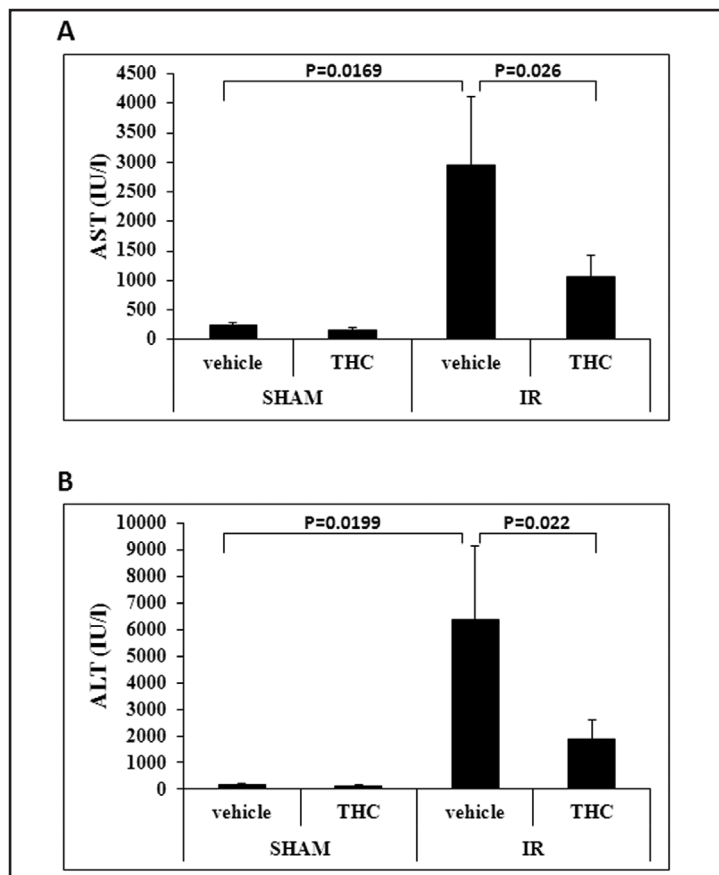
Improved hepatic histologic injury following the administration of THC
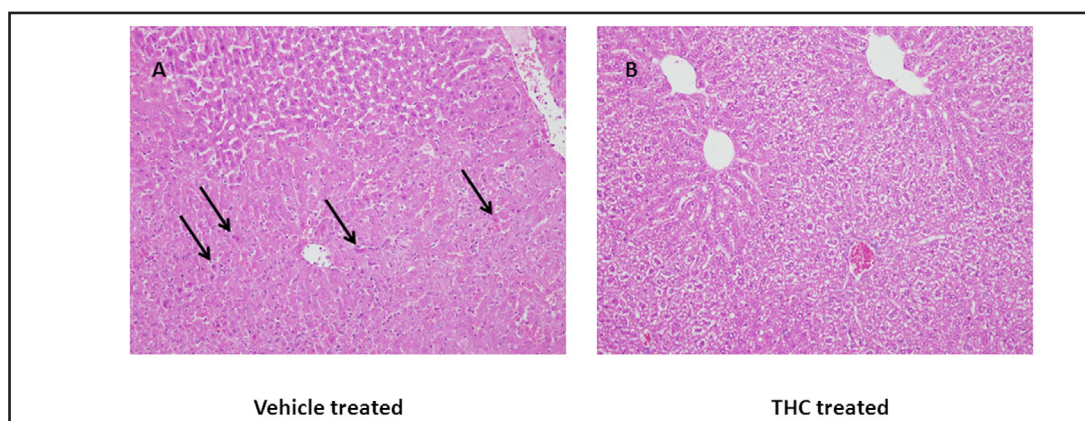
Histologic examination of the liver tissue sections using H&E staining showed damage in the ischemic untreated livers (group 3), manifested by diffused microvesicular vacuolization. Hydropic degeneration of hepatocytes was also noted and apoptotic bodies were found. In addition, we have detected focal necrosis infiltrated by neutrophils and mitotic figures (Fig. 2A). In the THC-pretreated group 4, the liver structure was better preserved and minimal vacuolization was noted. Rare apoptotic bodies and rare mitotic figures were noted (Fig. 2B).

Reduced hepatic apoptotic injury following the administration of THC

To identify the apoptotic effect of hepatic I/R, we performed analysis using the TUNEL assay. Apoptosis in the ischemic livers was confirmed by TUNEL assay. Sections from ischemic untreated livers in group 3 showed extensive DNA fragmentation as a fluorescent

Fig. 1. A statistically significant difference was noted in mean serum ALT/AST levels following the induction of I/R, compared with sham operated mice. In THC pretreated mice following the induction of I/R, the increase in serum liver enzymes level was significantly less compared with untreated mice. A statistically significant difference was noted in mean serum ALT/AST levels between the two untreated and I/R THC treated mice.

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Vehicle treated   THC treated

Fig. 2. Histologic examination of the liver tissue sections using H&E staining showed damage in the ischemic untreated livers (group 3), manifested by microvesicular vacuolization. Many hepatocytes in zones 2 and 3 were distended by lipid vacuoles (arrows). Hydropic degeneration of hepatocytes was also noted and apoptotic bodies (arrows) were found. In addition, we have detected focal necrosis infiltrated by neutrophils and mitotic figures (2A). In the THC-pretreated group 4, the liver structure was better preserved. Rare apoptotic bodies and rare mitotic figures were noted (2B).
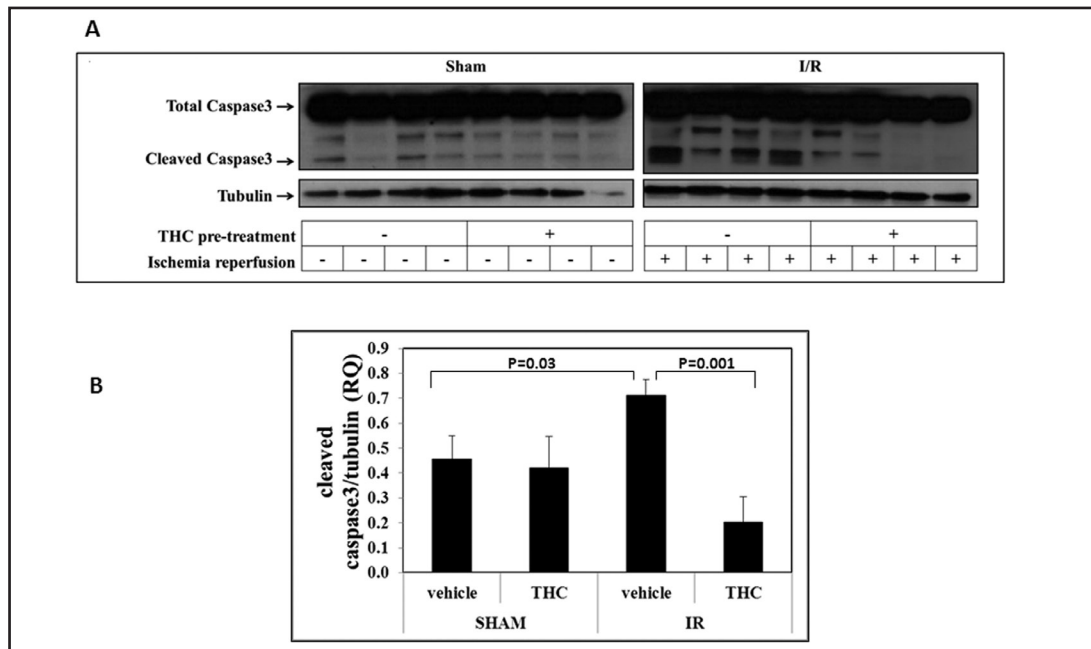
Fig. 3. Apoptosis in the ischemic livers was confirmed by the TUNEL assay. Sections from ischemic untreated livers in group 3 showed extensive DNA fragmentation as a fluorescent signal with intense staining (Fig. 3A). The intensity and quantity of fluorescence was significantly decreased in the ischemic THC-pretreated group (Fig. 3B), and minimal DNA fragmentation was noted. DAPI staining in all sections was intense indicating the presence of cells.
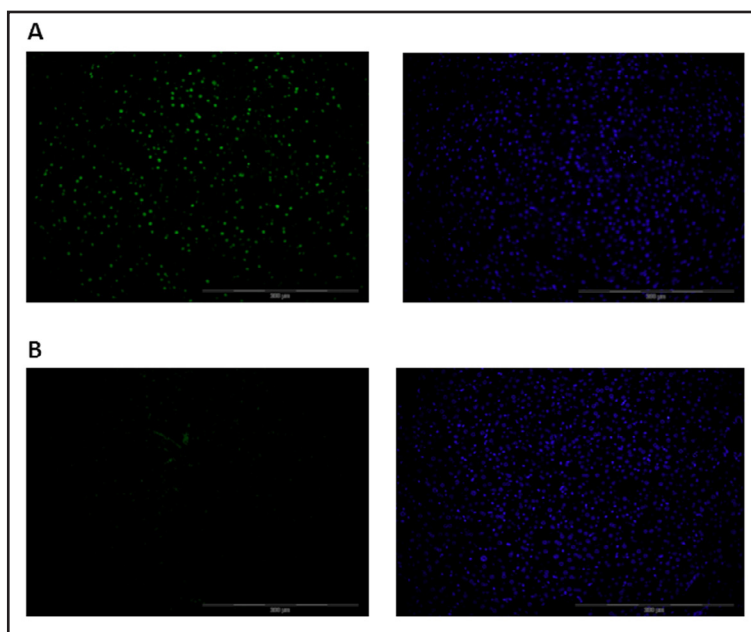
signal with intense staining (Fig. 3A). The intensity and quantity of fluorescence was significantly decreased in the ischemic THC-pretreated group (Fig. 3B), and minimal DNA fragmentation was noted. DAPI staining in all sections was intense indicating the presence of cells. In addition, the hepatic cleavage of the pro-apoptotic caspase-3 to its active form was significantly higher in the I/R untreated group compared with the Sham group however, there were lower levels of the cleaved caspase-3 in the THC treated mice compared with the untreated I/R group (Fig 4). The difference between the hepatic level of cleaved caspase-3 with or without THC pretreatment was statistically significant (p=0.01) (Fig 4).
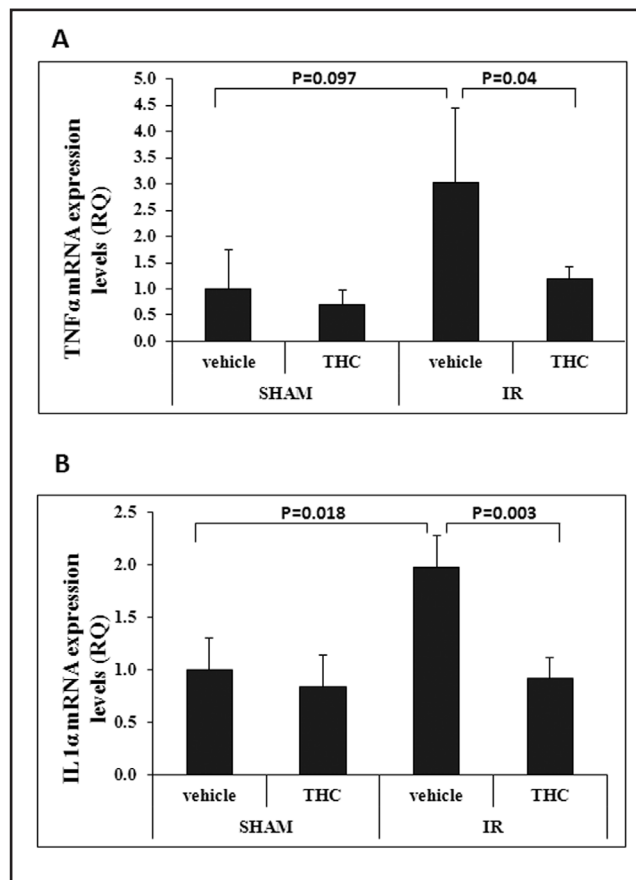
Reduced hepatic expression of the pro-inflammatory genes TNF-α and IL-1α following THC administration

The hepatic pro-inflammatory TNFα and IL1α genes were up-regulated in the I/R mice compared with the sham control group (5A, 5B). Pre-treatment with THC decreased

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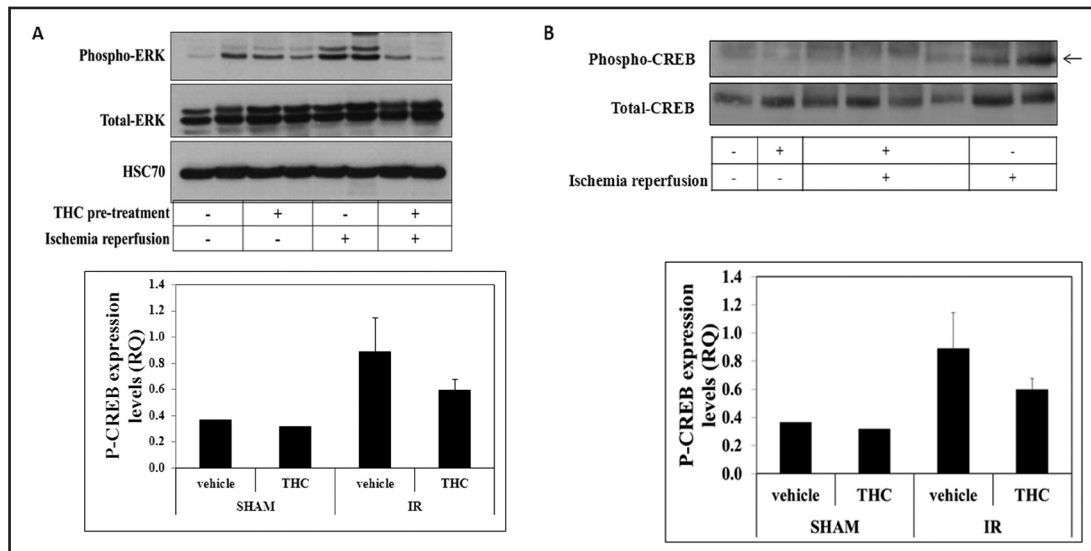Hochhauser et al.: Delta 9-Tetrahydrocannabinol in Hepatic Ischemia/Reperfusion Injury

Fig. 4. The hepatic cleaved pro-apoptotic caspase-3 activity was significantly higher in the I/R untreated group compared with the Sham group however, caspase-3 protein was significantly much less activated in the THC treated mice compare with the untreated I/R group.

Fig. 5. The hepatic pro-inflammatory TNFα and IL1α genes were up-regulated in the I/R mice compared with the sham control group (5A, 5B). Pre-treatment with THC decreased significantly the mRNA expression of both TNF-α and IL-1α genes. (5A, 5B).


significantly the mRNA expression of both TNF-α and IL-1α genes. (p=0.04 p= 0.0034, respectively) (5A, 5B).

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Fig. 6. Up-regulation in the hepatic phosphorylation level of ERK was noted in I/R livers but not in the I/R livers that were pre-treated with THC (6A). CREB phosphorylation was up-regulated in the I/R livers but not in the I/R livers that were pre-treated with THC (6B).

Fig. 7. Up-regulation in the hepatic in cFOS and IL-10 transcription level was noted following the administration of THC in I/R livers (7A, 7B).
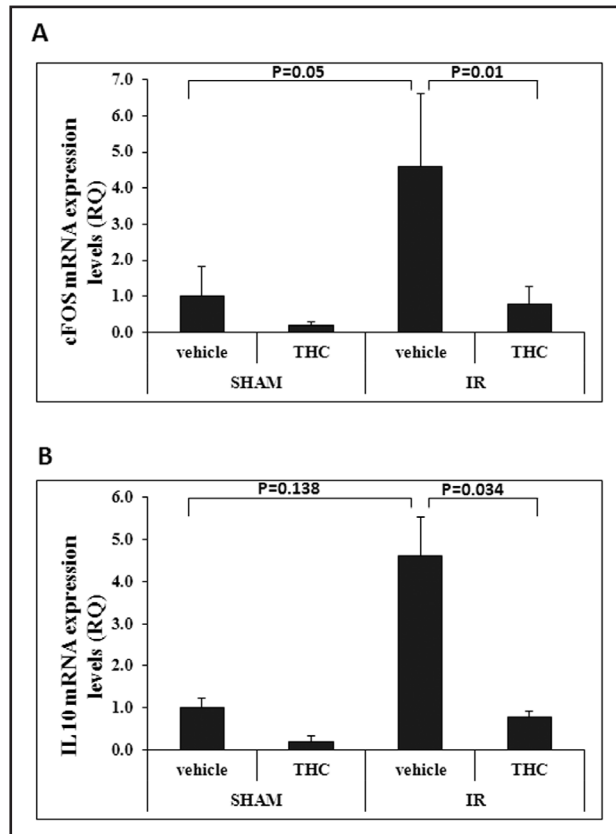
Hepatic ERK signaling pathway was suppressed following THC administration

Up-regulation in the hepatic phosphorylation level of ERK was noted in I/R livers but not in the I/R livers that were pre-treated with THC (Fig. 6A). One of the ERK signaling target is CREB. Upon ERK induction, CREB is phosphorylated and serves as a transcription factor for target genes. Two of the CREB target genes are cFOS and IL10. CREB phosphorylation was up-regulated in the I/R livers but not in the I/R livers that were pre-treated with THC

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(statistical analysis could not be performed due to the small numbers in each group) (Fig. 6B). A similar effect was noted in cFOS and IL-10 transcription level following the administration of THC in I/R livers (Fig. 7A and 7B). (cFOS p= 0.01, IL10 p=0.034).

Discussion

The present study demonstrates that a single application of an ultra-low dose of the cannabinoid agonist tetrahydrocannabinol (THC) to mice provides a significant protection against an ischemic insult to the liver. Pretreatment with 0.002 mg/kg of THC 2 h before the induction of hepatic I/R reduced significantly the necrotic and apoptotic injury 6 h post I/R, compared to vehicle-pretreatment. Furthermore, the inflammatory signaling pathways, oxidative stress, and cell death were all attenuated in mice pretreated with THC compared to vehicle-pretreated mice. Thus, a combined measurement of biochemical and morphological criteria indicated that THC conferred a significant hepatoprotection following I/R injury.

Hepatic I/R injury alter the mitochondrial function and generate reactive oxygen species during the reperfusion phase [2, 3]. Oxidative stress is increased due to Kupffer cell activation and, subsequently, inflammatory reactions, which, together with endothelial cell injury and microcirculatory dysfunction, may cause extensive areas of tissue necrosis. Several studies have shown that exposure to oxidative stress activates mitogen-activated protein kinases (MAPKs), which is crucial for determining cell fate in IRI cell events. Among these kinases, extracellular signal regulated kinase (ERK) inactivation has been implicated in protecting against hepatic I/R cell injury [15, 16] as we have shown in our study following the administration of ultra low dose THC. In a study by Bendinelli P et al, the MAP kinase cascades was activated during post-ischemic liver reperfusion [15]. Pan ZG, et al has demonstrated that bone marrow mesenchymal stem cells ameliorate hepatic I/R injuries via inactivation of the MEK/ERK signaling pathway in rats [16]. The intracellular signaling transcription factors cAMP response element-binding protein (CREB) is one of the targets of ERK signaling. Following the induction of ERK, CREB is phosphorylated and serves as a transcription factor for targeted genes. We have demonstrated in our study that following the induction of the I/R hepatic injury, that the hepatic oxidative stress induced the activation of CERB as was also demonstrated previously by Tacchini L et al [17]. In addition we have noticed increased hepatic expression of the proinflammatory cytokines TNF-α, IL-1α and of IL-10 and c-FOS that are CERB targeted genes. These findings were also reported previously in the perfusate of livers subjected to warm ischemia-reperfusion and in other several hepatic I/R studies [18-20].

In this and our previously published studies, we and others have suggested a major role also for apoptosis after hepatic I/R injury [21-26]. Rudiger et al. [22] reported that apoptosis is a central mechanism of cell death following reperfusion of the ischemic liver, and that apoptosis is associated with a caspase-dependent pathway [21, 22]. The apoptotic pathway in the ischemic liver is complex involving the release of tumor-necrosis factor-α, release of cytochrome c from the mitochondria resulting in activation of caspases and DNA fragmentation. We have also demonstrated in several studies that apoptosis is a central mechanism of cell death following reperfusion of the ischemic liver [23, 24]. For more than decay there is an ongoing controversy not yet solved regarding the mechanisms of cell death during hepatic I/R injury: necrosis or apoptosis [21]. A recently published study [27] in a murine model of hepatic I/R injury demonstrated extensive necrotic cell damage and inflammation by using several plasma biomarkers of apoptosis (CK18) and necrosis (FK18) together with histological evaluations. No evidence from morphological assessment, TUNEL staining, various plasma biomarkers, or pharmacological interventions with a pancaspase inhibitor suggested a relevant contribution of apoptotic cell death to the overall injury [27]. However, the use of CK18 as a serum biomarker of hepatic apoptosis following I/R was not evaluated or validated previously. Moreover, apoptotic cells in general disappear within a few hours which may lead to underestimation of apoptotic cell death. The TUNEL assay

utilized in our study is widely used for the evaluation of hepatic apoptosis as evident by numerous previous publications [22-26]. In addition, in contrast to the study by Yang et al [27], the administration of caspase inhibitors protected against hepatic I/R injury [26]. The debate is still ongoing; apoptosis could represent an early phase of the hepatic I/R injury. Necrosis and apoptosis might coexist after hypoxic liver injury reflecting shared mechanistic pathways [21].

Previous animal studies have already demonstrated hepatoprotective effects of various cannabinoid drugs [6-12, 25, 26]. It should be noted, however, that these studies presented a common experimental features: the doses that were administrated intraperitonealy ranged between 3 and 20 mg/kg (the doses that also induce the conventional acute effects of cannabinoids). Such high doses may not only induce unwanted cannabinoid effects but may also activate non-selective pathways that are not mediated by the specific cannabinoid receptors [26, 27]. This parameter might be of clinical importance. We previously showed that the same ultra-low dose of THC conferred a significant neuroprotection when applied to mice either 7 days before or 3 days after brain insult [10]. In addition we have demonstrated that a single ultra low dose of THC before ischemia is a safe and effective treatment that reduces myocardial ischemic damage when applied up to 48 hours before the insult [13]. We have previously found that the single application of 0.002 mg/kg of THC to mice induced long-lasting activation of protective signaling molecules in the brain, including the transcription factor CREB and the trophic factor BDNF (brain derived neurotophin [12]. These finding suggested that the ultra-low dose of THC may stimulate an endogenous adaptive mechanism in addition to the well documented acute anti-inflammatory effect of cannabinoids when applied in high doses.

Recent data have shown that CB2 receptors decrease the extent of liver injury in models of acute liver insult, as induced by I/R [6-9]. In mice, segmental ischemia followed by reperfusion (but not ischemia alone) markedly increased the hepatic levels of arachidonoyl ethanolamide (AEA), also known as anandamide and 2-arachidonoyl glycerol (2-AG), endogenous ligands, correlated with the severity of tissue damage [6]. I/R-induced tissue damage, including neutrophil infiltration and lipid peroxidation, was attenuated by pretreatment with JWH-133 (activator of CB2 cannabinoid receptors) in wild-type mice but not in CB2 -/- mice, in which the damage was more severe than that in wild-type littermates [6]. Another potent and selective CB2 agonist, HU-308, caused similar effects and also attenuated I/R-induced hepatocyte apoptosis and mitigated the TNFα-induced expression of cell adhesion molecules (intercellular cell adhesion molecule 1 and vascular cell adhesion molecule 1) in hepatic sinusoidal endothelial cells [8]. All these effects were previously attributed to the wellknown immunomodulatory activity of CB2 receptors.

We have found in the present study that ultra low dose THC, given prior to the induction of I/R, significantly attenuated the elevations of serum liver transaminases ALT and AST (markers of necrotic liver injury), the hepatic oxidative (activation of the intracellular signaling CREB pathway), the enhanced acute proinflammatory response (TNF-α, IL-1α, IL-10, and c-FOS hepatic mRNA levels, and ERK signaling pathway activation) at 6 hours of reperfusion. This was followed by cell death (hepatic caspase 3 cleavage, DNA fragmentation DAPI activity, and TUNEL) at 6 hours of reperfusion. As THC is a mixed CB1/CB2 agonist, its hepatoprotective profile may share some features with the above mentioned CB2 agonists. However, our preliminary experiments (Sarne et al, unpublished data) indicate that this ultra-low dose of THC is devoid of any direct immunomodulatory activity. In light of the long lasting activation by this low dose of THC of signaling pathways in the brain that are involved in cell survival (12), it is feasible that similar effects take place in the liver and that THC may provide a wider spectrum of hepatoprotection.

A future examination of the dose-response relationships may indicate whether even lower doses of THC are hepatoprotective and whether even more effective protection can be achieved. Experiments with specific antagonists may reveal which receptor is involved and may pave the way to examine other, more selective cannabinoid agonists.

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In conclusion, our study provides novel evidence for the beneficial use of extremely low doses of THC, doses that do not elicit any psychoactive side effects, in order to protect the liver from ischemic insults. A single ultra low dose THC can reduce the apoptotic and inflammatory injury induced by hepatic I/R injury. THC may serve as a potential target for therapeutic intervention in hepatic I/R injury during liver transplantation, liver resection and trauma.

Disclosure Statement

None.

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