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Themed Section: Cannabinoids in Biology and Medicine, Part II

RESEARCH PAPER

Δ^8-Tetrahydrocannabivarin prevents hepatic ischaemia/reperfusion injury by decreasing oxidative stress and inflammatory responses through cannabinoid CB_2 receptors

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BACKGROUND AND PURPOSE

Activation of cannabinoid CB_2 receptors protects against various forms of ischaemia-reperfusion (I/R) injury. Δ^8-Tetrahydrocannabivarin (Δ^8-THCV) is a synthetic analogue of the plant cannabinoid Δ^9-tetrahydrocannabivarin, which exhibits anti-inflammatory effects in rodents involving activation of CB_2 receptors. Here, we assessed effects of Δ^8-THCV and its metabolite 11-OH-Δ^8-THCV on CB_2 receptors and against hepatic I/R injury.

EXPERIMENTAL APPROACH

Effects *in vitro* were measured with human CB_2 receptors expressed in CHO cells. Hepatic I/R injury was assessed in mice with 1h ischaemia and 2, 6 or 24h reperfusion *in vivo*.

KEY RESULTS

Displacement of [^{3}H]CP55940 by Δ^8-THCV or 11-OH-Δ^8-THCV from specific binding sites in CHO cell membranes transfected with human CB_2 receptors (hCB_2) yielded K_i values of 68.4 and 59.95 nM respectively. Δ^8-THCV or 11-OH-Δ^8-THCV inhibited forskolin-stimulated cAMP production by hCB_2 CHO cells (EC_{50} = 12.95 and 14.3 nM respectively). Δ^8-THCV, given before induction of I/R, attenuated hepatic injury (measured by serum alanine aminotransferase and aspartate aminotransferase levels), decreased tissue protein carbonyl adducts, 4-hydroxy-2-nonenal, the chemokines CCL3 and CXCL2,TNF-α, intercellular adhesion molecule 1 (CD54) mRNA levels, tissue neutrophil infiltration, caspase 3/7 activity and DNA fragmentation. Protective effects of Δ^8-THCV against liver damage were still present when the compound was given at the beginning of reperfusion. Pretreatment with a CB_2 receptor antagonist attenuated the protective effects of Δ^8-THCV, while a CB_1 antagonist tended to enhance it.

CONCLUSIONS AND IMPLICATIONS

Δ^8-THCV activated CB_2 receptors *in vitro*, and decreased tissue injury and inflammation i*n vivo*, associated with I/R partly via CB_2 receptor activation.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2012.165.issue-8. To view Part I of Cannabinoids in Biology and Medicine visit http://dx.doi.org/10.1111/bph.2011.163.issue-7

Abbreviations

4-HNE, 4-hydroxy-2-nonenal; Δ^8-THCV, Δ^8-tetrahydrocannabivarin; AEA, anandamide (*N*-arachidonoyl ethanolamine); CB_1 or CB_2 receptor, cannabinoid 1 or 2 receptor; ICAM-1, intercellular adhesion molecule 1 (CD54); I/R, ischaemia/reperfusion; ROS, reactive oxygen species; RNS, reactive nitrogen species

Introduction

Transient ischaemia followed by reperfusion (I/R) is a principal mechanism of organ injury in common pathological conditions, such as myocardial infarction and stroke, and may accompany surgical interventions involving vascular occlusion. In particular, solid organ transplantation inherently exposes the graft to some degree of I/R injury, which considerably affects outcome. The deleterious effects of I/R arise from the acute generation of reactive oxygen (ROS) and nitrogen species (RNS) (Ferdinandy and Schulz, 2003; Pacher *et al.*, 2007) 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 (Liaudet *et al.*, 2003; Pacher and Hasko, 2008).

Δ^8-Tetrahydrocannabivarin (O-4395, Δ^8-THCV) is a synthetic analogue of Δ^9-tetrahydrocannabivarin (Δ^9-THCV), a plant cannabinoid, which is a propyl homologue of Δ^9-tetrahydrocannabinol, the main psychoactive ingredient of marijuana (Gill *et al.*, 1970). Initial pharmacological experiments have already suggested that the plant-derived Δ^9-THCV was a possible ligand for cannabinoid (CB) receptors (see Pertwee, 2008). It has been shown that Δ^9-THCV may act as a CB_1 receptor antagonist (Thomas *et al.*, 2005; Pertwee *et al.*, 2007; Ma *et al.*, 2008; Pertwee, 2008; receptor nomenclature follows Alexander *et al.*, 2011); however, it can also activate CB_2 receptors (Bolognini *et al.*, 2010), as well as non-CB_1, non-CB_2 receptor targets (see Pertwee, 2008). Studies using Δ^8-THCV have shown that it has a similar pharmacological profile to Δ^9-THCV. Thus, both compounds behave as CB_1 receptor antagonists *in vitro* and *in vivo* at low doses. However, Δ^8-THCV may also exhibit agonistic activity *in vivo* at higher doses (Pertwee *et al.*, 2007). Regarding CB_2 receptors, evidence from a very recent study implies that Δ^9-THCV may behave as partial agonists at CB_2 receptors, not only *in vitro*, but also *in vivo*, as shown by reduced inflammation and inflammatory pain in mouse models (Bolognini *et al.*, 2010). The rationale behind the development of Δ^8-THCV is that it is more stable than the plant-derived analogue Δ^9-THCV, and it is easier to synthesize.

The protective role of CB_2 receptor activation in a well-characterized mouse model of hepatic I/R injury has previously been demonstrated (Batkai *et al.*, 2007; Rajesh *et al.*, 2007; Moon *et al.*, 2008). In the current study, we have investigated the effects of Δ^8-THCV on the course of liver damage, oxidative stress, and acute and chronic inflammatory response and cell death. In our model, tissue damage is induced by the generation of reactive oxygen species (ROS) that is followed by a cascade of acute and chronic inflammatory response. Because a recent study has found that Δ^9-THCV displayed high selectivity and potency as a CB_2 receptor agonist (Bolognini *et al.*, 2010), and CB_2 receptors on endothelial, inflammatory and perhaps some parenchyma cells mediate protective effects in hepatic (Batkai *et al.*, 2007; Rajesh *et al.*, 2007), cardiac (Pacher and Hasko, 2008; Defer *et al.*, 2009; Montecucco *et al.*, 2009) and cerebral (Zhang *et al.*, 2007; 2009a,b; Murikinati *et al.*, 2010) I/R injury (Pacher and Mechoulam, 2011), we also explored the plausible role of CB_2 receptors in the effects of Δ^8-THCV on hepatic I/R injury. Our findings reinforce the potential of Δ^8-THCV for prevention/treatment of hepatic, and perhaps other forms of, I/R injury.

Materials and methods

CHO cells

CHO cells stably transfected with cDNA encoding human cannabinoid CB_2 receptors (B_{max} = 72.6 pmol·mg^{-1} protein) were maintained at 37°C and in 5% CO_2 in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM supplemented with 1 mM L-glutamine, 10% fetal calf serum, 0.6% penicillin–streptomycin, hygromycin B (300 μg·mL^{-1}) and G418 (400 μg·mL^{-1}). After reaching a confluent state, the cells were washed with PBS and passaged using non-enzymic cell dissociation fluid.

Membrane preparation

Binding assays with [^{3}H]CP55940 were performed with CHO–hCB_2 cell membranes as described by Ross *et al.* (1999). The hCB_2-transfected cells were removed from flasks by scraping and then frozen as a pellet at –20°C until required. Before using in a radioligand binding assay, the cells were defrosted, diluted in 50 mM Tris binding buffer and homogenized with a 1 mL handheld homogenizer. Protein assays were performed using a Bio-Rad Dc kit (Bio-Rad, Hercules, CA, USA).

Radioligand displacement assay

The assays were carried out with [^{3}H]CP55940, Tris binding buffer (50 mM Tris–HCl, 50 mM Tris–base, 0.1% BSA), total assay volume 500 μL, using the filtration procedure described previously by Ross *et al.* (1999). Binding was initiated by the

addition of CHO–hCB$_2$ cell membranes (50 μg protein per well). All assays were performed at 37°C for 60 min before termination by the addition of ice-cold Tris binding buffer and vacuum filtration using a 24-well sampling manifold (cell harvester, Brandel Inc., Gaitherburg, MD, USA) and GF/B filters (Whatman, Maidstone, UK) that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 mL aliquot of Tris binding buffer. The filters were oven-dried for 60 min and then placed in 5 mL of scintillation fluid (Ultima Gold XR, PerkinElmer Life Sciences Inc., Waltham, MA, USA). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabelled CP55940. The concentration of [^{3}H]CP55940 used in our displacement assays was 0.7 nM. Δ^8-THCV and 11-OH-Δ^8-THCV were stored as stock solutions of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. These were diluted with Tris binding buffer before use. Drug additions were made in a volume of 10 μL to produce a final vehicle concentration in all assay wells of 0.1% DMSO. The binding parameters for [^{3}H]CP55940, determined by fitting data from saturation binding experiments to a one-site saturation plot using GraphPad Prism (San Diego, CA, USA), were 72 570 fmol·mg^{-1} protein (B_{max}) and 1.043 nM (K_d) as reported previously (Thomas *et al.*, 2005).

cAMP assay

Adherent CHO–hCB$_2$ cells were washed once with Dulbecco's PBS and detached using non-enzymic cell dissociation solution. After centrifugation, the cells were resuspended (2×10^6 cells·mL^{-1}) in buffer containing PBS (calcium and magnesium free), 1% BSA and 10 μM rolipram. The cells were incubated for 30 min at 37°C with the cannabinoid under investigation. A further 30 min of incubation was carried out with 10 μM of forskolin in a total volume of 500 μL. The reaction was terminated by the addition of 500 μL of 0.1 M HCl, followed by centrifugation to remove cell debris. The pH was then adjusted to between 8.0 and 9.0 by the addition of 1 M of NaOH, and cyclic AMP content was measured using a radioimmunoassay kit (GE Healthcare, Amersham Ltd., Little Chalfont, Buckinghamshire, UK). Forskolin and rolipram were dissolved in DMSO and stored at –20°C as 10 mM stock solutions.

Metabolism of AEA and 2-AG in vitro

The synthesis of AEA through the activity of *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) was assayed using brain homogenates (50 μg per test) and 100 μM [^{3}H]NArPE as reported (Maccarrone *et al.*, 2008). The hydrolysis of AEA by fatty acid amide hydrolase (FAAH) was assessed in brain homogenates (20 μg per test), by measuring the release of [^{3}H]arachidonic acid from 10 μM [^{3}H]AEA, as reported (Maccarrone *et al.*, 2008). NAPE-PLD activity was expressed as pmol of AEA released per min per mg of protein, whereas FAAH activity was expressed as pmol of arachidonate released per min per mg of protein. The synthesis of 2-AG through the activity of diacylglycerol lipase (DAGL) was assayed with 10 μM [^{14}C]DAG as substrate, and that of the 2-AG hydrolase, monoacylglycerol lipase (MAGL), was determined using 10 μM [^{3}H]2-OG as substrate, as reported (Maccarrone *et al.*, 2008). Both DAGL and MAGL activities were expressed as pmol product per min per mg protein. In all cases, the effect of Δ^8-THCV on enzyme activity was ascertained by adding this substance directly to the assay buffer.

Hepatic I/R

All animal care and experimental procedures in this study complied with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees of NIAAA. Male C57BL/6J mice (25–30 g; Jackson Laboratories, Bar Harbor, ME, USA) were anaesthetized with pentobarbital sodium (65 mg·kg^{-1} i.p.). In our study, the model of segmental (70%) hepatic ischaemia has been used, as described (Batkai *et al.*, 2007; Rajesh *et al.*, 2007; Moon *et al.*, 2008). Briefly, the liver was exposed by midline laparotomy, and the hepatic artery and the portal vein were clamped using an atraumatic micro-serrefine. This method of partial ischaemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The duration of hepatic ischaemia was 60 min, after which the vascular clips were removed and liver was reperfused for 2, 6 or 24 h, as indicated. Sham surgeries were identical except that hepatic blood vessels were not clamped with a micro-serrefine. The liver was kept moist at 37°C with gauze soaked in 0.9% saline. Body temperature was monitored with a rectal temperature probe and was maintained at 37°C by a heating blanket. Treatment with Δ^8-THCV 3 and 10 mg·kg^{-1} or vehicle i.p., started 2 h before IR or were given post-ischaemia at the moment of reperfusion, as indicated in the text. CB$_1$ and CB$_2$ antagonists were given 90 min prior to ischaemia or Δ^8-THCV pretreatment. At the experimental end points, blood was collected and liver samples were removed and snap-frozen in liquid nitrogen for determining biochemical parameters or fixed in 4% buffered formalin for histopathological evaluation (Batkai *et al.*, 2007; Rajesh *et al.*, 2007; Moon *et al.*, 2008).

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels

The activities of AST and ALT, indicators of liver cellular damage, were measured in serum samples using a clinical chemistry analyser system (VetTest 8008, IDEXX laboratories, Westbrook, ME, USA) (Batkai *et al.*, 2007; Rajesh *et al.*, 2007; Moon *et al.*, 2008).

Histological examination of liver sections

Liver samples were fixed in 4% buffered formalin. After embedding and cutting 5 μm slices, all sections were stained with haematoxylin/eosin (HE). Myeloperoxidase (MPO) in neutrophils was stained with an anti- MPO antibody according to the manufacturer's protocol (Biocare Medical, Concord, CA, USA), and samples were counter-stained with nuclear fast red as described earlier (Batkai *et al.*, 2007; Rajesh *et al.*, 2007). Histological evaluation was performed without knowledge of the treatments .

Real-time PCR analyses of mRNA

Total RNA was isolated from liver homogenate using TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolated RNA was treated

with RNase-free DNase (Ambion, Austin, TX, USA) to remove traces of genomic DNA contamination. One microgram of total RNA was reverse-transcribed to cDNA using the Super-Script II (Invitrogen). The target gene expression was quantified with Power SYBER Green PCR Master Mix using an ABI HT7900 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Each amplified sample in all wells was analysed for homogeneity using dissociation curve analysis. After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s, and at 60°C for 30 s. Relative changes in mRNA levels were calculated using the comparative C_t method. This method involves comparing the C_t values of the samples of interest with a control (2-$\Delta\Delta C_t$ method: $\Delta\Delta C_t = \Delta C_t$ sample – ΔC_t reference). Lower ΔC_t values and lower $\Delta\Delta C_t$ reflect a relatively higher amount of gene transcript (Schmittgen and Livak, 2008). Statistical analyses were carried out for at least 6 to 15 replicate experimental samples in each set.

The primers used were as follows:

CCL3, 5′-TGCCCTTGCTGTTCTTCTCTG-3′ and 5′-CAA CGATGAATTGGCGTGG-3′; CXCL2, 5′-AGTGAACTGCGCT GTCAATGC-3′ and 5′-AGGCAAACTTTTTGACCGCC-3′; TNF-α, 5′-AAGCCTGTAGCCCACGTCGTA-3′ and 5′-AGGTA CAACCCATCGGCTGG-3′; ICAM-1, 5′-AACTTTTCAGCTCCG GTCCTG-3′ and 5′-TCAGTGTGAATTGGACCTGCG-3′; and actin, 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GGATGCAG GGATGATGTTC-3′

Hepatic 4-hydroxy-2-nonenal (4-HNE) content

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as 4-HNE, which has been shown to be capable of binding to proteins and forming stable HNE adducts. HNE in hepatic tissues was determined using a kit (Cell Biolabs, San Diego, CA, USA). In brief, BSA or hepatic tissue extracts (10 μg·mL^{-1}) were adsorbed onto a 96-well plate for 12 h at 4°C. HNE adducts present in the sample or standard were probed with anti-HNE antibody, followed by an HRP conjugated secondary antibody. The HNE protein adduct content in an unknown sample was determined by comparing with a standard curve (Mukhopadhyay *et al.*, 2010).

Detection of hepatic carbonyl adducts

Carbonyl content in liver tissues was determined by OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs) (Rajesh *et al.*, 2010; Mukhopadhyay *et al.*, 2011). In brief, BSA standards or protein samples (10 μg·mL^{-1}) were adsorbed onto a 96-well plate for 2 h at 37°C. The protein carbonyls present in the sample or standard were derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in the unknown sample was determined by comparing with a standard curve that was prepared from predetermined reduced and oxidized BSA standards.

Detection of apoptosis by caspase 3/7 activity assays

Caspase 3/7 activity in hepatic tissue lysate was measured using the Apo-One Homogenous Caspase-3/7 assay kit (Promega Corp., Madison, WI, USA). An aliquot of caspase reagent was added to each well and mixed on a plate shaker for 1 h at room temperature shielded from light, and the fluorescence was measured (Mukhopadhyay *et al.*, 2009; Rajesh *et al.*, 2009).

Hepatic DNA fragmentation ELISA

The quantitative determinations of cytoplasmic histone-associated DNA fragmentation (mono- and oligonucleosomes) due to cell death in liver homogenates were measured using ELISA kit (Roche Diagnostics Corp., Indianapolis, IN, USA) (Mukhopadhyay *et al.*, 2009; Pan *et al.*, 2009).

Statistical analysis

Values have been expressed as means and variability as SEM or as 95% confidence limits. The concentrations of Δ^8-THCV and 11-OH-Δ^8-THCV that produced a 50% displacement of radioligand from specific binding sites (IC_{50} values) were calculated using GraphPad Prism 5. Their dissociation constants (K_i values) were calculated using the equation of Cheng and Prusoff (1973). GraphPad Prism 5 was also used to calculate EC_{50} and E_{max} values from data obtained in the binding or cyclic AMP assays. Statistical significance among groups was determined by one-way ANOVA followed by Newman–Keuls *post hoc* analysis using GraphPad Prism 5 software. Probability values of $P < 0.05$ were considered significant.

Materials

Δ^8-THCV (6a*R*,10a*R*)-6,6,9-trimethyl-3-propyl-6a,7,10,10a-tetrahydro-6*H*-benzo[c]chromen-1-ol or O-4395) and 11-OH-Δ^8-THCV were synthesized by Dr Anu Mahadevan (Organix Inc, Woburn, MA, USA). Δ^8-THCV has been synthesized as follows. *p*-Menth-2-ene-1,8-diol (Figure 1A, compound **1**) (3.98 g, 23.5 mmol) and 5-propylbenzene-1,3-diol (Figure 1A, compound **2)** (3.2 g, 21.3 mmol) were dissolved in dry benzene (300 mL) and *p*-toluene sulphonic acid (200 mg, 1.1 mmol) was added. The reaction mixture was refluxed for 4 h using a Dean–Stark apparatus, cooled to room temperature and stirred overnight. The reaction mixture was concentrated under vacuum, and the crude material was purified by silica gel column chromatography eluting with 7% ethyl acetate/hexane to yield Δ^8-THCV as a light brown solid (Figure 1A).

Forskolin and rolipram were purchased from Sigma-Aldrich (Poole, Dorset, UK), CP55940 (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol from Tocris (Bristol, UK) and [^{3}H]CP55940 from PerkinElmer Life Sciences Inc. (Boston, MA, USA). CB_1 and CB_2 antagonists/inverse agonists SR141716A and SR144528 were obtained from the NIDA Drug Supply Program. For *in vivo* administration, all drugs were dissolved in vehicle solution (1 drop of Tween-80 in 3 mL 2.5% DMSO in saline). Vehicle solution was used in control experiments.

Anandamide (*N*-arachidonoyl ethanolamine, AEA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and 2-arachidonoyl glycerol (2-AG) was from Alexis Corporation (San Diego, CA, USA). [^{3}H]AEA (205 Ci·mmol^{-1}) was from PerkinElmer Life Sciences. [^{3}H]*N*-arachidonoyl phosphatidylethanolamine ([^{3}H]NArPE, 200 Ci·mmol^{-1}), and [^{3}H]2-oleoyl glycerol ([^{3}H]2-OG, 20 Ci·mmol^{-1}) were from

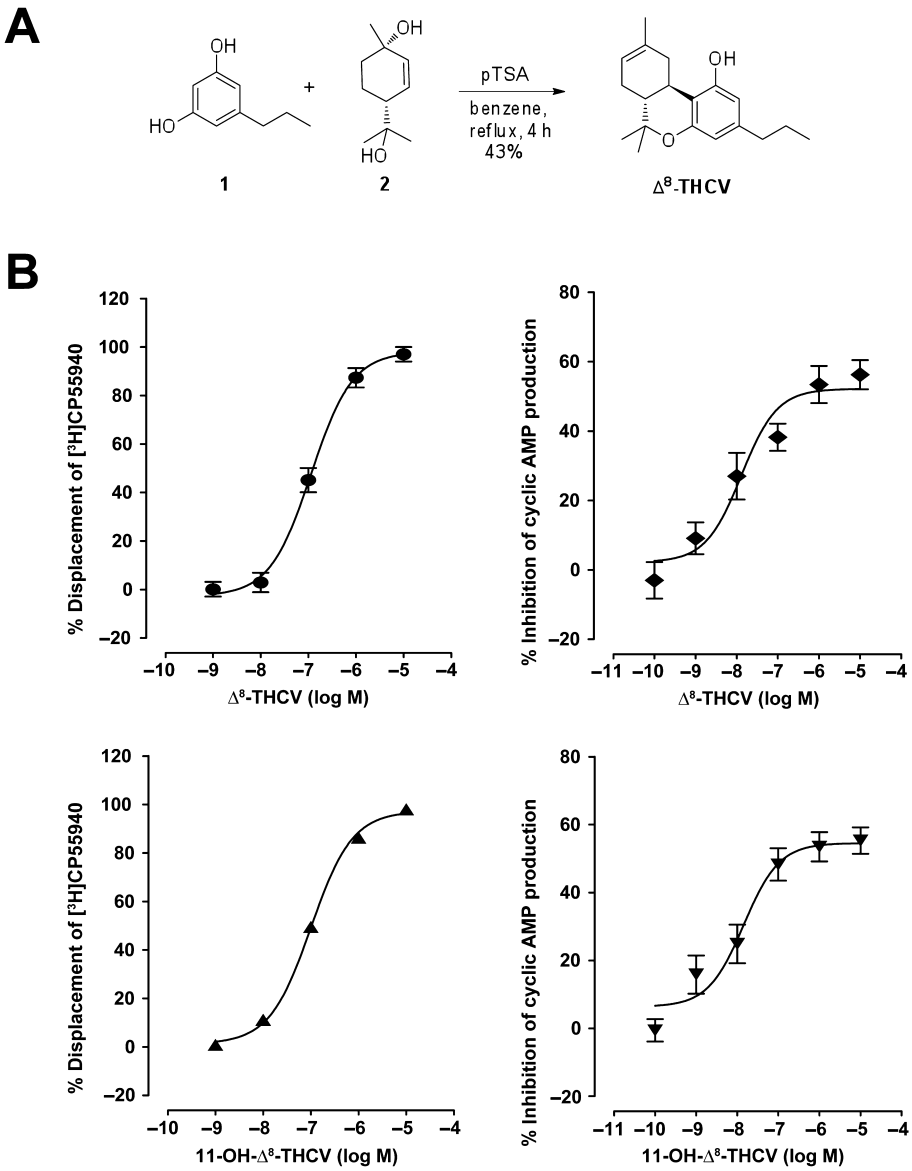

Figure 1

Synthesis and *in vitro* effects of Δ^8-THCV on CB_2 receptors. (A) Scheme of the synthesis of Δ^8-THCV from 5-propylbenzene-1,3-diol (1) and *p*-menth-2-ene-1,8-diol (2). (B) Left-hand panels: displacement of [^{3}H]CP55940 from specific binding sites in CHO hCB_2 cell membranes. Each symbol represents the mean percent displacement ± SEM (n = 6). Right-hand panels: inhibition of forskolin-induced stimulation of cyclic AMP production in CHO hCB_2 cells. Each symbol represents the mean percent inhibition ± SEM (n = 7). The derived K_i, EC_{50} and E_{max} values are given in the Results.

ARC (Arlington, VA, USA), whereas [^{14}C]*sn*-1-stearoyl-2-arachidonoyl glycerol ([^{14}C]DAG, 56 mCi·mmol^{-1}) was from Amersham (Glattbrugg, Switzerland).

Results

Δ^8-THCV and 11-OH-Δ^8-THCV activate CB_2 receptors in vitro

Mean K_i values for displacement of [^{3}H]CP55940 from specific binding sites in CHO hCB_2 cell membranes with 95% confidence limits shown in brackets were 68.4 nM (46.2 and 101.3 nM; n = 6) for Δ^8-THCV, and 59.95 nM (49.1 and 73.2 nM; n = 6) for 11-OH-Δ^8-THCV (Figure 1B). Corresponding E_{max} values were 98.0% (91.4 and 104.7%) and 97.3% (94.2 and 100.4%) respectively. Δ^8-THCV and 11-OH-Δ^8-THCV inhibited forskolin-induced stimulation of cyclic AMP production in CHO hCB_2 cells with mean EC_{50} values of 12.95 nM (4.5 and 37.6 nM; n = 7) for Δ^8-THCV, and 14.3 nM (5.3 and 38.7 nM; n = 7) for 11-OH-Δ^8-THCV (Figure 1B). Corresponding E_{max} values were 52.2% (45.6 and 58.8%) and 54.6% (48.2 and 61.0%) respectively.

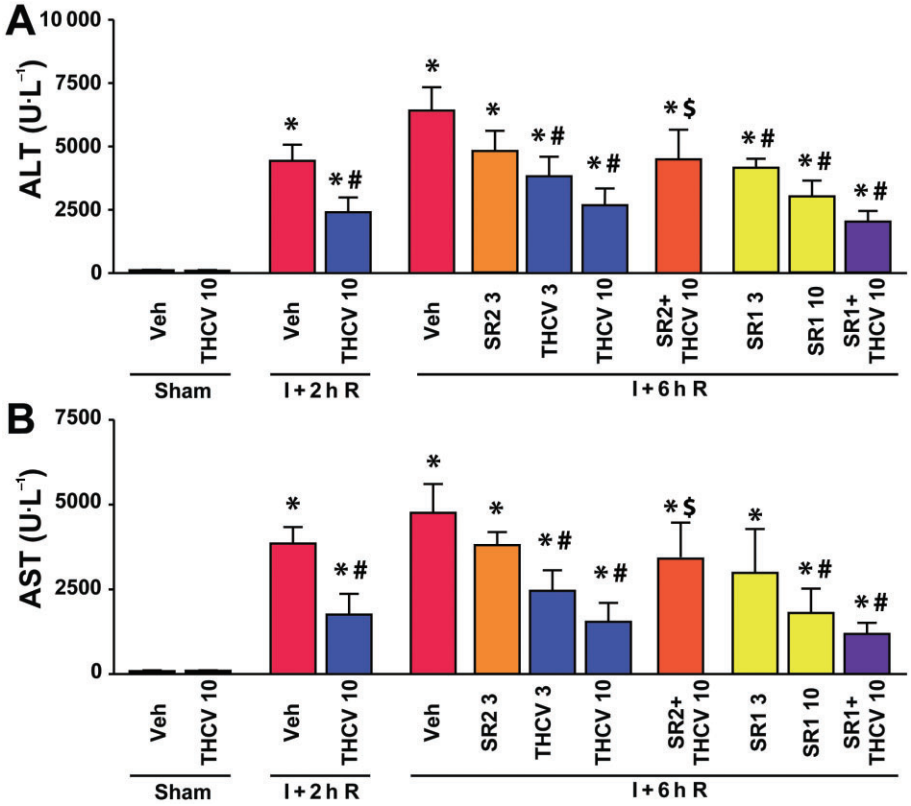

Figure 2

Δ^8-THCV pretreatment decreases liver I/R injury. (A and B) Serum transaminase ALT (A) and AST (B) levels in sham-operated mice treated with vehicle (veh) or Δ^8-THCV ($n = 5$ per group) or in mice exposed to 1 h of hepatic ischaemia followed by 2h (I + 2h R) or 6 h (I + 6h R) of reperfusion pretreated with vehicle, Δ^8-THCV (3 or 10 mg·kg^{-1}) or SR144528 (SR2, 3 mg·kg^{-1}), SR141716A (SR1, 3 and 10 mg·kg^{-1}) and 10 mg·kg^{-1} Δ^8-THCV in combination with SR1 or SR2 ($n = 6$–10 per group). *$P < 0.05$ versus vehicle; #$P < 0.05$ versus corresponding mice exposed to I/R; \$$P < 0.05$ versus corresponding mice exposed to I/R and pretreated with 10 mg·kg^{-1} Δ^8-THCV.

Δ^8-THCV does not affect the activity of enzymes involved in endocannabinoid synthesis and hydrolysis

In a set of *in vitro* experiments, 10 μM Δ^8-THCV almost halved NAPE-PLD activity (466 ± 56 vs. 833 ± 33 pmol·min^{-1}·mg^{-1} protein, $P < 0.001$), whereas at the same concentration, it did not change the FAAH activity (125 ± 5 pmol·min^{-1}·mg^{-1} protein in both Δ^8-THCV-treated and control samples). In addition, 2-AG synthesis *via* DAGL was not significantly affected by 10 μM Δ^8-THCV (396 ± 126 vs. 273 ± 103 pmol·min^{-1}·mg^{-1} protein in both Δ^8-THCV-treated and control samples; $P > 0.05$), neither was 2-AG degradation *via* MAGL (689 ± 16 vs. 653 ± 19 pmol·min^{-1}·mg^{-1} protein in Δ^8-THCV-treated and control samples; $P > 0.05$). Taken together, these data demonstrate that Δ^8-THCV does not affect the metabolism of AEA or 2-AG.

Δ^8-THCV attenuates markers of hepatic I/R injury (ALT, AST)

For assessments of hepatocellular damage of the post-ischaemic liver, the serum transaminase activities (AST and ALT) were measured. After 1 h of ischaemia and a subsequent 2 or 6 h of reperfusion, a dramatic increase in liver enzyme activities was observed in vehicle-treated C57BL/6J mice as compared with sham-operated controls (Figure 2). Pretreatment with Δ^8-THCV (3 or 10 mg·kg^{-1}) 2 h before the induction of the ischaemia dose-dependently attenuated the serum transaminase elevations at 2 and 6 h of reperfusion compared to vehicle. At 2 h reperfusion with 10 mg·kg^{-1} Δ^8-THCV, ALT level decreased by ~46% and AST by ~54%. At 6 h reperfusion with 3 or 10 mg·kg^{-1} Δ^8-THCV, ALT decreased by 41 and 58%, respectively, whereas AST levels decreased by 48 and 69%, respectively (Figure 2). Pretreatment with the CB_2 receptor antagonist/inverse agonist SR144528 (3 mg·kg^{-1}) alone had no statistically significant effect on post-ischaemic transaminase levels; however, it attenuated the beneficial effect of 10 mg·kg^{-1} Δ^8-THCV at the time of the peak serum ALT/AST elevations (Figure 2), implying a role for CB_2 receptors in the protective effects of Δ^8-THCV. On the other hand, the CB_1 antagonist/inverse agonist SR141716 pretreatment alone at the doses of 3 and 10 mg·kg^{-1} attenuated the injury (Figure 2). Also SR141716 in combination with 10 mg·kg^{-1} Δ^8-THCV produced a stronger protective effect, although it did not reach statistical significance. Δ^8-THCV, SR141716 or SR144528 alone had no effects on ALT and AST levels in the sham animals compared to the vehicle-treated group.

Post-ischaemic Δ^8-THCV treatment, i.e., given at the moment of reperfusion attenuated (Figure 3), although to a lesser extent than in the pretreatment experiments, the hepatic injury measured at 6 h of reperfusion.

Δ^8-THCV improves I/R-induced histological damage

HE staining of representative liver sections after 24 h of reperfusion showed (Figure 4) that I/R induced marked coagulation necrosis (lighter areas, with marked inflammatory cell infiltration), which was dramatically reduced and became more focal in Δ^8-THCV-treated mice. Vehicle or Δ^8-THCV treatment in the sham animals had no effect on liver histopathology. A similar histological profile was seen throughout the groups (n = 3–5).

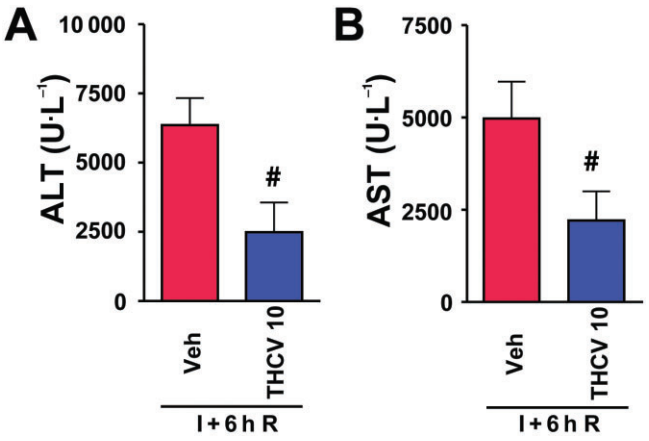

Figure 3

Δ^8-THCV treatment at the moment of reperfusion decreases liver I/R injury. (A and B) Serum transaminase ALT (A) and AST (B) levels in mice exposed to 1 h of hepatic ischaemia followed by 6 h of reperfusion (I+6h R) treated with vehicle or Δ^8-THCV (10 mg·kg^{-1}, n = 7–8 per group) at the start of reperfusion. #P < 0.05 versus corresponding mice exposed to I/R.

Δ^8-THCV attenuates the I/R-induced marked neutrophil infiltration

Neutrophils are important mediators of the delayed tissue injury following I/R. An indicator of neutrophil infiltration is the tissue MPO activity. In sham mice, MPO staining was barely detectable (Figure 5). In contrast, there was a marked increase in infiltrating MPO-positive immune cells (brown staining), after 24 h of reperfusion in vehicle-treated animals, which was significantly attenuated by Δ^8-THCV. A similar histological profile was seen throughout the groups (n = 3–5).

Δ^8-THCV attenuates the I/R-induced hepatic pro-inflammatory chemokine, cytokines and adhesion molecule expression

I/R greatly increased the expression of mRNAfor the chemokines CCL3 and CXCL2 in liver tissue as shown by real-time PCR, which was attenuated by Δ^8-THCV pretreatment (Figure 6A,B). Hepatic CCL3 mRNA increased to the highest level at 2 h of reperfusion and gradually decreased by 24 h of reperfusion almost to control levels. Δ^8-THCV pretreatment decreased hepatic peak CCL3 mRNA level at 2 and 6 h of reperfusion (Figure 6A). mRNA for CXCL2 increased to its peak at 2 h of reperfusion and gradually decreased by 24 h of reperfusion (Figure 6B). Δ^8-THCV treatment significantly decreased peak levels of mRNA for CCL3 and CXCL2 at 2 h of reperfusion, and also markedly attenuated the I/R values at 6 h of reperfusion (Figure 6A,B).

Real-time PCR analyses of cytokine TNF-1α (Figure 6C) and adhesion molecule ICAM-1 (Figure 6D) demonstrated markedly increased mRNA expression in liver tissue after I/R,

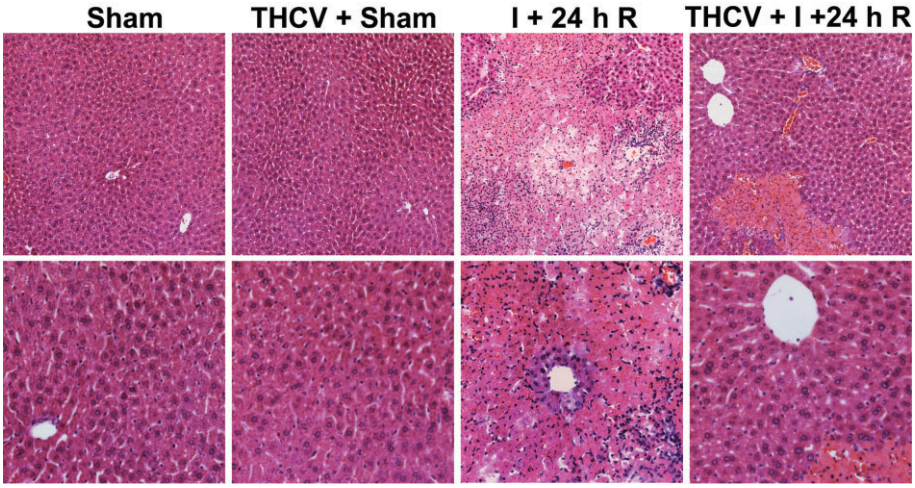

Figure 4

Δ^8-THCV decreases histological damage at 24 h following ischaemia. HE staining of representative liver sections of sham mice treated with vehicle (sham) or Δ^8-THCV (THCV), and mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated with vehicle (I + 24 h R) or Δ^8-THCV (THCV + I + 24 h R). Upper row of images depicts 200× magnification, while the lower one 400× magnification. A similar histological profile was seen throughout each group (n = 3–5).

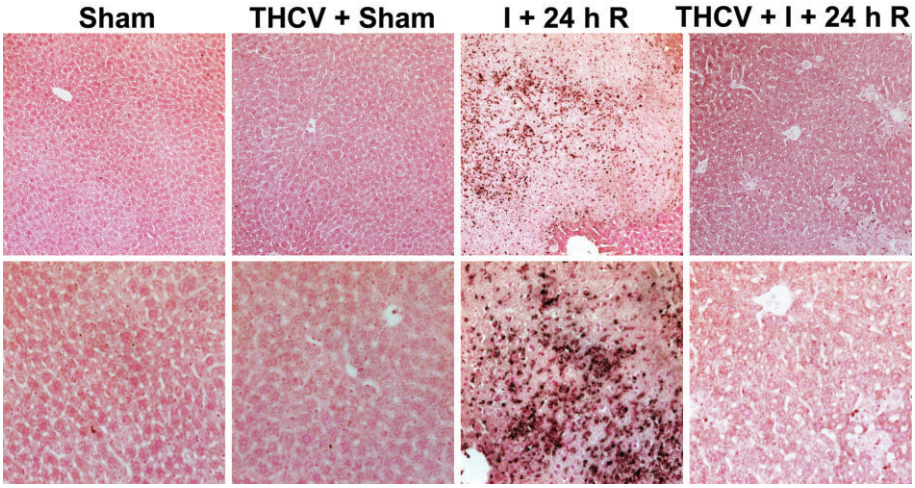

Figure 5

Δ^8-THCV decreases neutrophil infiltration. MPO staining (brown) of representative liver sections of sham mice treated with vehicle (sham) or Δ^8-THCV (THCV), and mice exposed to 1 h of ischaemia followed by 24 h of reperfusion treated with vehicle (I + 24 h R) or Δ^8-THCV (THCV + I + 24 h R). Slides were counterstained by nuclear fast red. Upper row of images depicts 200× magnification, while the lower one 400× magnification. A similar histological profile was seen throughout each group (n = 3–5).

peaking at 2 h of reperfusion and gradually declining thereafter. Δ^8-THCV pretreatment significantly decreased the I/R-induced increased TNF-α and ICAM-1 mRNA expression, and also markedly attenuated these values at 6 h of reperfusion (Figure 6C,D).

Δ^8-THCV decreases the I/R-induced increased oxidative stress

The rate of oxidative post-translational modification of proteins and lipid peroxidation was negligible in sham livers, as indicated by the low level of carbonyl adducts and HNE. HNE increased by 2.5- and 4-fold after 6 and 24 h of reperfusion, respectively, and these increases were significantly attenuated by Δ^8-THCV (10 mg·kg^{-1}) (Figure 7A). The hepatic content of carbonyl adducts increased by 3.2- and 7.1-fold after 6 and 24 h of reperfusion, respectively, which were significantly attenuated by Δ^8-THCV (10 mg·kg^{-1}) (Figure 7B).

Δ^8-THCV decreases the I/R-induced hepatocyte cell death

Apoptotic cell death in the liver following I/R was evaluated by monitoring caspase-3/7 activity and DNA fragmentation (Figure 8). All markers of cell death in the liver were increased at 24 h of reperfusion following 60 min ischaemia. Δ^8-THCV significantly attenuated the increase of both markers (Figure 8).

Discussion

In the present study, we have demonstrated that Δ^8-THCV exerted protective effects against liver I/R reperfusion damage by attenuating tissue injury, oxidative stress and inflammatory response. Hepatic I/R injury may occur in a number of clinical settings, such as those associated with low flow states, surgical procedures and transplantation, affecting morbidity and mortality. The damage to the liver caused by I/R represents a continuum of processes that culminate in tissue injury. These processes are triggered when the liver is transiently deprived of oxygen and subsequently re-oxygenated. The destructive effects of I/R are induced by the rapid generation of ROS from the activation of various cellular sources, such as xanthine oxido-reductases (Engerson *et al.*, 1987; Pacher *et al.*, 2006) and impairment of the mitochondrial respiratory chain (Jaeschke, 2003; Moon *et al.*, 2008), resulting in increased lipid peroxidation, oxidative modification of proteins and DNA damage, impairing pivotal cellular functions. The increased ROS during early reperfusion also leads to activation of Kupffer cells (the resident inflammatory cells of the liver) and endothelial cells with consequent increased production of pro-inflammatory chemokines (this response peaks 2 h following reperfusion) and cytokines in these cells, leading to the priming and recruitment of neutrophils and other inflammatory cells into the liver vasculature upon reperfusion. These inflammatory cells attach to the activated endothelium and generate ROS and RNS, and pro-inflammatory mediators leading to endothelial dysfunction, followed by transmigration through the injured endothelium, attachment to hepatocytes and further activation to release oxidants and proteolytic enzymes. These changes in turn trigger intracellular oxidative/nitrative stress and mitochondrial dysfunction in hepatocytes (the oxidative/nitrative stress peaks at 24 h of reperfusion), culminating in cell death (both apoptotic and necrotic) (Jaeschke, 2006; Pacher and Hasko, 2008).

In agreement with this cascade of pathological events and previous reports using the same model (Batkai *et al.*, 2007; Rajesh *et al.*, 2007; Moon *et al.*, 2008), we have found increased hepatic carbonyl and 4-HNE adduct levels (markers of lipid peroxidation and oxidative stress), and increased serum ALT and AST activities (markers of liver injury/

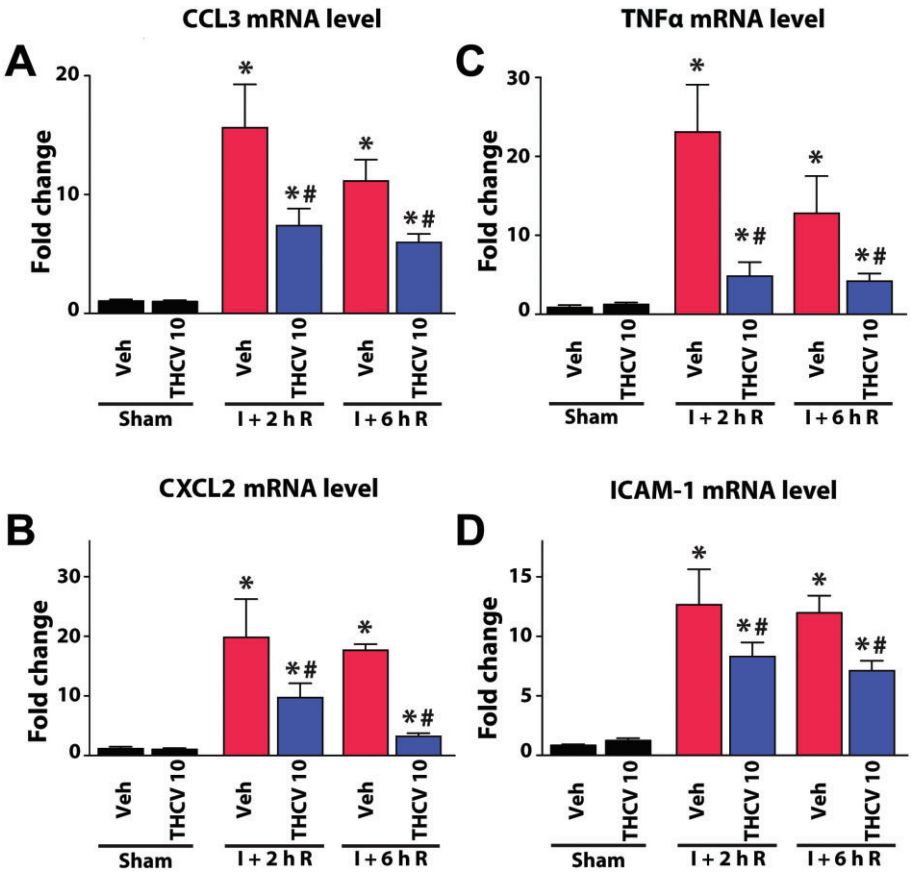

Figure 6

Δ^8-THCV attenuates the I/R-induced acute pro-inflammatory response in the liver. Real-time PCR shows significant increase of mRNA for the chemokine CCL3 (A), the chemokine CXCL2 (B), the pro-inflammatory cytokine TNF-α (C) and adhesion molecule ICAM-1 (D) at 2 h of reperfusion (I + 2 hR), and a gradual decrease at 6 h (I + 6 hR). Pretreatment with Δ^8-THCV at 10 mg·kg^{-1} significantly attenuates the I/R-induced increased levels of these inflammatory markers at the time points of the reperfusion studied (2 and 6 h). Results are mean ± SEM; n= 6–12. *P < 0.05 versus vehicle; #P < 0.05 versus corresponding I/R mice.

necrosis) at 2 h of reperfusion, accompanied by peak increases in markers of acute inflammatory response and endothelial activation [mRNA for TNF-α, CCL3, CXCL2, ICAM-1 (CD54)] in the liver. The peak levels of serum ALT and AST could be detected at 6 h of reperfusion in our model, which gradually returned to almost normal levels by 24 h of reperfusion, indicating that the predominant type of cell death at the earlier time points of reperfusion is necrotic. As the inflammatory reactions progress, the histological picture of post-ischaemic liver morphology at 24 h of reperfusion is characterized by marked coagulation necrosis (lighter areas) with massive inflammatory cell infiltration (Figures 4 and 5). In this model of I/R, MPO-positive neutrophil recruitment starts from 6 h of reperfusion and peaks during 12–24 h of reperfusion (Moon *et al.*, 2008) (Figure 5), which coincides with the peak of oxidative stress and apoptotic cell death (caspase 3/7 activity and DNA fragmentation) 24 h following reperfusion (Figures 7 and 8).

Δ^8-THCV is a synthetic analog of Δ^9-THCV, a natural component of marijuana. The latter has been shown to exert protective effects against seizure activity (Hill *et al.*, 2010); has also hypophagic properties; and is in phase I development as a potential treatment for obesity, diabetes and related metabolic disorders (Riedel *et al.*, 2009). On the other hand, Δ^9-THCV also exhibits significant potency and efficacy as a CB_2 receptor agonist *in vivo*, and exerts anti-oedema and anti-hyperalgesic activity in a model of local inflammation (Bolognini *et al.*, 2010). Based on these data, we hypothesized that Δ^8-THCV may also activate CB_2 receptors and mediate beneficial effects in hepatic I/R.

Indeed, we found that Δ^8-THCV and one of its metabolites, 11-OH-Δ^8-THCV (Harvey and Brown, 1988), behaved as potent CB_2 receptor agonists *in vitro*. In our binding assay, Δ^8-THCV caused agonist displacement in hCB_2-transfected CHO cells and also inhibited forskolin-induced stimulation of cAMP production (with an EC_{50} of 13 nM and E_{max} of 52%) in these cells, which were not significantly different from the recently reported values for Δ^9-THCV (EC_{50} = 38 nM and E_{max} = 40%; Bolognini *et al.*, 2010).

Various cannabinoid ligands may also interact with the synthetic and metabolic enzymes of endocannabinoids, eliciting indirect effects mediated by changes of the levels of these bioactive substances, with potential relevance to tissue injury (Pacher and Mechoulam, 2011). However, we found

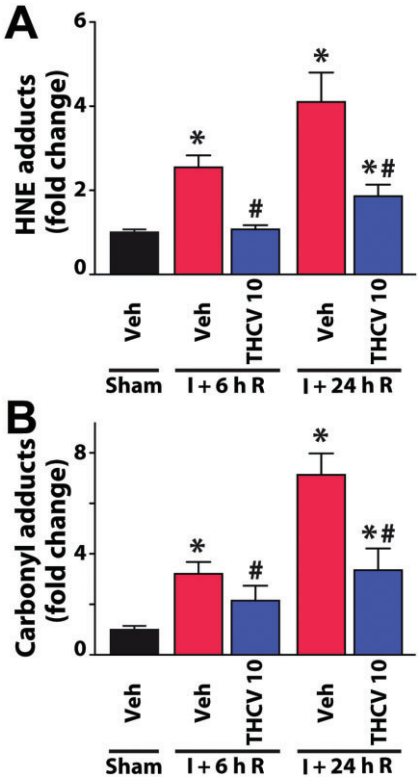

Figure 7

Δ^8-THCV decreases I/R-induced increased oxidative stress. (A) HNE adducts, a marker for lipid peroxidation/oxidative stress, increase with time following I/R injury. 10 mg·kg^{-1} Δ^8-THCV pretreatment attenuates these increases at the time points of reperfusion studied, 6h (I + 6h R) and 24 h (I+24h R). (B) Oxidative modification of proteins, measured by carbonyl adducts, increases with time following I/R injury. 10 mg·kg^{-1} Δ^8-THCV pretreatment attenuates these increases at the time points of reperfusion studied, 6h (I + 6h R) and 24 h (I+24h R). Results are mean ± SEM for both panels and $n = 8$ per group. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus corresponding I/R mice.

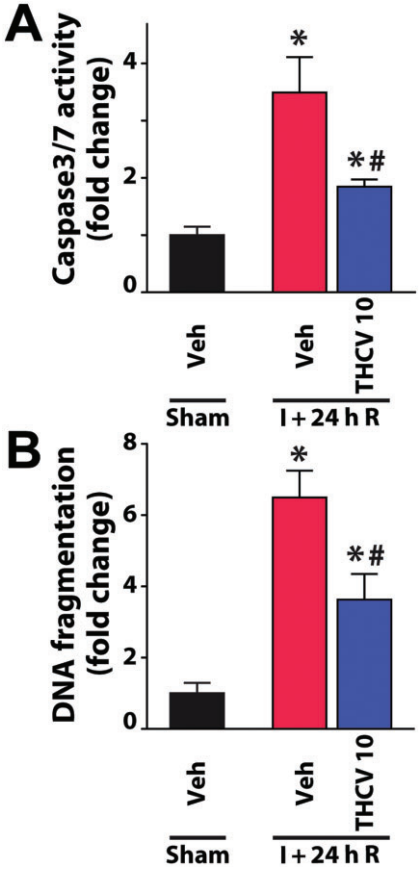

Figure 8

Δ^8-THCV attenuates the I/R-induced enhanced hepatic cell death. (A) Changes in hepatic caspase3/7 activity following I/R, and attenuation of the observed increases by pretreatment with 10 mg·kg^{-1} Δ^8-THCV. Results are mean ± SEM of $n = 8$ per group. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus corresponding I/R mice. (B) Increase of hepatic DNA fragmentation demonstrated following I/R injury and attenuation by pretreatment with 10 mg·kg^{-1} Δ^8-THCV. Results are mean ± SEM of $n = 8$ per group. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus corresponding I/R mice.

that Δ^8-THCV in relevant concentrations had no effect on the activities of the major enzymes involved in endocannabinoid synthesis (NAPE-PLD and DAGL) or degradation (FAAH and MAGL). Although in this study we have not tested the effect of Δ^8-THCV on the putative endocannabinoid transporter(s), on the basis of the data described earlier, it is unlikely that Δ^8-THCV would directly lead to major changes in tissue endocannabinoid levels in normal livers. However, it is very likely that by decreasing inflammation and oxidative stress during I/R, which are involved in modulating endocannabinoid production/degradation (Pacher and Hasko, 2008), Δ^8-THCV may indirectly affect tissue endocannabinoid levels in damaged tissues, an interesting issue which should be explored in future studies.

In our *in vivo* model of liver I/R injury, Δ^8-THCV, given prior to the induction of I/R, significantly attenuated the elevations of serum liver transaminases (ALT/AST), decreased tissue oxidative stress (carbonyl adducts and HNE), attenuated acute and chronic hepatic inflammatory response (TNF-α, CCL3, CXCL2, ICAM-1/CD54 mRNA levels and tissue neutrophil infiltration), and necrotic (ALT/AST levels and coagulation necrosis) and apoptotic (caspase 3/7 activity, DNA fragmentation) cell death. The protective effects of Δ^8-THCV were largely abolished by pretreatment with the CB_2 receptor antagonist SR144528, indicating that these were, at least in part, mediated by CB_2 receptor activation (CB_2 antagonist in tested dose had no significant effect on I/R injury by itself). Pretreatment with the CB_1 receptor antagonist SR141716 did not prevent the beneficial effect of Δ^8-THCV, in fact it showed protective effects by itself, which tended to be more pronounced when Δ^8-THCV was given in combination with SR141716. The protective effect of the CB_1 receptor antagonist observed in our study is consistent with the protection observed in rat liver I/R complicated with endotoxemia recently (Caraceni *et al.*, 2009) and in other models of I/R injury (Pacher and Hasko, 2008; Zhang *et al.*, 2009b), likewise with the proposal of combining CB_2 receptor agonists with CB_1 receptor antagonists for the treatment of

reperfusion damage (Pacher and Hasko, 2008; Zhang *et al.*, 2009b). From this perspective, it would be interesting to see in future studies if Δ^8-THCV could inhibit CB_1 receptors in *in vitro* and *in vivo* models of injury. Importantly, the protective effects of Δ^8-THCV against liver damage were also preserved when it was given after the ischaemia, at the moment of reperfusion, which is very important from a clinical point of view.

In summary, our results indicate that Δ^8-THCV, a more stable and more readily synthesized analogue of the naturally occurring Δ^9-THCV, may represent a novel protective strategy against I/R injury by attenuating oxidative stress, acute and chronic inflammatory response, and cell death.

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Conflict of interest

R.G.P. receives funding from GW Pharmaceuticals; other authors have no conflicts.

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