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Pivotal Advance: Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis

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Abstract

In this study, we have investigated the role of the cannabinoid CB_2 (CB_2) receptor in an in vivo mouse model of hepatic ischemia/reperfusion (I/R) injury. In addition, we have assessed the role of the CB_2 receptor in TNF-α-induced ICAM-1 and VCAM-1 expression in human liver sinusoidal endothelial cells (HLSECs) and in the adhesion of human neutrophils to HLSECs in vitro. The potent CB_2 receptor agonist HU-308, given prior to the induction of I/R, significantly attenuated the extent of liver damage (measured by serum alanine aminotransferase and lactate dehydrogenase) and decreased serum and tissue TNF-α, MIP-1α, and MIP-2 levels, tissue lipid peroxidation, neutrophil infiltration, DNA fragmentation, and caspase 3 activity. The protective effect of HU-308 against liver damage was also preserved when given right after the ischemic episode. HU-308 also attenuated the TNF-α-induced ICAM-1 and VCAM-1 expression in HLSECs, which expressed CB_2 receptors, and the adhesion of human neutrophils to HLSECs in vitro. These findings suggest that selective CB_2 receptor agonists may represent a novel, protective strategy against I/R injury by attenuating oxidative stress, inflammatory response, and apoptosis.

Keywords

endothelial activation; adhesion; inflammation; TNF-α

INTRODUCTION

Ischemia reperfusion (I/R) is the major mechanism of organ injury during myocardial infarction, stroke, coronary bypass surgery, and organ transplantation. The devastating effects of I/R arise from the acute generation of reactive oxygen and nitrogen species following reoxygenation, which inflict direct tissue injury and initiate a chain of deleterious cellular responses leading to inflammation, cell death, and eventually, organ failure (reviewed in refs. [1–7]).

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The endocannabinoid system is a novel target in various inflammatory, metabolic, cardiovascular, gastrointestinal, and liver disorders (reviewed in refs. [8–15]). The synthetic and natural ligands (the latter called endocannabinoids: arachidonoyl ethanolamide or anandamide and 2-arachidonoylglycerol) of cannabinoid (CB) receptors exert various anti-inflammatory and neuroprotective [16–18] effects by inhibiting the generation and release of proinflammatory cytokines and mediators (reviewed in refs. [14,19–21]). Two CB receptors have been identified by molecular cloning to date: the CB_1 receptor, which is highly expressed in the brain [22] but is also present in peripheral tissues, including the heart [23,24], vascular tissues [25,26], and liver [27–30], and the CB_2 receptor, previously thought to be expressed primarily in immune and hematopoietic cells (ref. [31]; reviewed in ref. [14]). More recent studies have also identified CB_2 receptors in brain [32], myocardium [33], cardiomyoblasts [33,34], and endothelial cells of various origins [35–38].

There is limited and conflicting information about the role of CB receptor activation in cell-protective mechanisms against tissue damage (including I/R) in the heart and brain (reviewed in refs. [14,19,20,39–41]). Most published studies were performed by using ex vivo models (e.g., isolated perfused hearts), where the role of important interactions of activated endothelium and leukocytes and other immunomodulatory effects of CBs could not be evaluated, and used nonspecific ligands for CB_2 receptors (reviewed in refs. [14,39–42]).

In the present study, we have used a potent and selective CB_2 receptor agonist HU-308 [43] to reveal the role of CB_2 receptors in an in vivo model of liver ischemia reperfusion and in TNF-α-induced ICAM-1 and VCAM-1 expression in human liver sinusoidal endothelial cells (HLSECs) and adhesion of human neutrophils to HLSECs in vitro.

MATERIALS AND METHODS

Animals

All animal experiments conformed to National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (NIAAA; Bethesda, MD, USA). C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

Hepatic I/R protocol

Mice were anesthetized with pentobarbital (65 mg/kg i.p.). A midline laparotomy incision was performed to expose the liver. The hepatic artery and the portal vein were clamped using microaneurysm clamps. This model results in a segmental (70%), hepatic ischemia. This method of partial ischemia prevents mesenteric, venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver, which was kept moist at 37°C with gauze soaked in 0.9% saline. Body temperature was maintained at 37°C using a thermoregulatory heating blanket and monitoring of body temperature with a rectal temperature probe. Sham surgeries were identical, except that hepatic blood flow was not reduced with a microaneurysm clamp, and animals were killed 3 h or 25 h following the surgery, respectively. The duration of hepatic ischemia was 60 min in all experiments, after which the microaneurysm clamps were removed. The duration of the reperfusion was 120 min or 24 h, as indicated. After reperfusion, blood was collected, and liver samples were removed, weighed, and snap-frozen in liquid nitrogen for determining biochemical parameters or fixed in 4% buffered formalin for histopathological evaluation.

Drugs

AM251 and AM630 were from Tocris (Baldwin, MO, USA), HU-308 was from Cayman Europe (Estonia). All drugs were dissolved in vehicle solution (one drop of Tween-80 in 3 ml

2.5% DMSO in saline) and injected i.p. 60 min prior the occlusion of the hepatic artery and the portal vein. In a separate set of experiments, HU-308 (10 mg/kg) was injected into the femoral vein right before the reocclusion. Vehicle solution was used in control experiments. For cell culture experiments, all lipid-soluble drugs were dissolved in DMSO. All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), except where it was mentioned.

Plasma alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels

The concentrations of ALT and LDH, indicators of liver and tissue damage, were measured in plasma samples using a clinical chemistry analyzer system (Prochem-V, Drew Scientific, Oxford, CT, USA), as described [44].

Lipid peroxidation

Malondialdehyde (MDA) formation was used to quantify the lipid peroxidation in tissues and measured as thiobarbituric acid-reactive material, as described [44,45]. Briefly, tissues were homogenized (100 mg/ml) in 1.15% KCl buffer. Homogenates (200 μl) were then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 μl 8.1% SDS, 1.5 ml 20% acetic acid (pH 3.5), and 600 μl distilled H_2O and heated at 90°C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000 *g*, 10 min), and their absorbance at 532 nm (A_{532}) was measured with 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nmol MDA/mg protein.

Myeloperoxidase (MPO) activity

MPO activity was measured as described previously [44,45]. Briefly, liver samples were homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-(N-morpholino)propanesulfonic acid and centrifuged to separate the supernatant. An aliquot of the supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM hydrogen peroxide. Activity was measured spectrophotometrically as the change in A_{650} at 37°C using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA). MPO activity was expressed as mU/mg protein. Protein content was determined with the *DC* protein assay (BioRad, Hercules, CA, USA).

Levels and expression of TNF-α, MIP-1α, MIP-2, and ICAM-1

The levels of the inflammatory cytokine TNF-α and the chemokines MIP-1α and MIP-2 in plasma or homogenized liver tissue were determined using commercially available ELISA (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol, as described previously [44,45].

DNA fragmentation assay

The assay was based on measuring the amount of mono- and oligonucleosomes in the cytoplasmic fraction of tissue extracts using the commercially available kit (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions, as described [33].

Caspase 3 activity

Caspase 3 activity in the tissue extracts was determined by using the commercially available kit (Chemicon, El Segundo, CA, USA). In brief, the assay is based on measuring the amount of chromophore *p*-nitroaniline (*p*NA) liberated from the labeled substrate Asp-Glu-Val-Asp-*p*NA at 405 nM as described [33].

Cell culture

HLSECs were obtained from Cell Systems (Kirkland, WA, USA) and grown in CSC-complete growth medium, according to the manufacturer's recommendations. Cells were grown in 0.2% gelatin-coated 100 mm cell culture dishes and used within three to six passages. Polymorphonuclear neutrophil leukocytes (PMN) were isolated from whole blood obtained from a healthy volunteer (NIH Clinical Center, Bethesda, MD, USA) using Percol (GE Biosciences, Piscataway, NJ, USA) density gradient solution.

RT-PCR analysis for CB_2 receptor gene expression was performed from HLSECs, as described previously [33].

Cell surface ICAM-1 and VCAM-1 expression

Cell surface expression of ICAM-1 and VCAM-1 was measured by in situ ELISA, as described [44]. In brief, HLSECs were grown in 96-well plates coated with 0.2% gelatin. The cells were treated with TNF-α (50 ng/ml) ± HU-308 (0–4 μM) for 4 h. In some experiments, the cells were treated with HU-308 (3 μM), AM251, or AM630, each used at the concentration of 1 μM for 1 h, followed by incubation with TNF-α for 4 h (indicated drugs were also present during TNF-α exposure). Then, cells were washed with PBS and fixed in 4% formaldehyde (pH 7.4) and blocked with PBS containing 1% BSA containing 100 mM glycine for 2 h at 4° C. The fixed monolayer was probed with anti-human ICAM-1 or VCAM-1 mAb (R&D Systems) for 1 h at 37°C and incubated with peroxidase-coupled anti-mouse IgG (1:5000, Pierce, Rockford, IL, USA) for 1 h at 37°C. Following washing, cells were incubated with 100 μl developing substrate solution (3,3′,5,5′-tetramethylbenzidine, Sigma Chemical Co.) for 10 min, and the reaction was terminated with 50 μl 2 N H_2SO_4, measured as A_{450}. Each treatment was performed in triplicate, and the experiments were repeated three times.

PMN—endothelial cell adhesion

Neutrophil adhesion to endothelial cells was performed as described [44]. In brief, HLSECs were grown to confluence in 24-well plates and treated with TNF-α ± HU-308 or pretreated with CB_1/CB_2 antagonists followed by treatment with TNF-α/HU-308 as described above. Then, PMN were labeled with 2.5 μM Calcein-AM (Molecular Probes-Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C in RPMI 1640 containing 1% FBS. HLSECs were washed twice with HLSEC basal medium and covered with 400 μl HLSEC basal medium. Then, 5×10^4/100 μl-labeled PMN cells were added to HLSECs and incubated for 1 h at 37°C. After incubation, the monolayer was washed carefully with PBS to remove the unbound PMN. The adherent PMN were documented by an Olympus IX 81 fluorescent microscope using a 20× objective (Opelco, Dulles, VA, USA). Three fields were captured per experimental condition. Individual treatments were preformed in duplicate, and the entire set of experiments was repeated twice. The number of adherent PMN cells was counted using NIH Image J software, and the values were expressed as PMN adhered/field.

Histological analysis of liver samples

Liver samples were fixed in 4% buffered formalin. After embedding and cutting 5 μm slices, all sections were stained with H&E. MPO staining of neutrophils was done by using anti-MPO antibody, according to the manufacturer's protocol (Zymed Lab., San Francisco, CA, USA), and samples were contrastained with nuclear fast red. Histological evaluation was performed in a blinded manner.

Statistical analysis

Results are presented as means ± SEM. One-way ANOVA followed by Newman-Keuls multiple comparisons post hoc analysis was calculated using the GraphPad Prism 4 package (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

RESULTS

CB_2 agonist attenuates markers of I/R-induced tissue injury (ALT and LDH), lipid peroxidation, and MPO activity

For assessment of tissue damage of the postischemic liver, the serum transaminase ALT and LDH activities were measured. After 60 min of ischemia and a subsequent 120-min reperfusion (60/120 min I/R), there was a dramatic increase in serum ALT and LDH activities in vehicle-treated C57Bl6/J mice as compared with sham-operated controls (Fig. 1, A and B). Pretreatment with 10 mg/kg CB_2 agonist HU-308 significantly reduced the transaminase and LDH level following I/R, and this effect was not prevented by the CB_1-selective antagonist AM251 but was attenuated significantly by the CB_2 antagonist AM630. HU-308 (10 mg/kg) also significantly attenuated the I/R-induced tissue damage when administered right before the 120-min reperfusion [ALT: 5338±631 vs. 3351±570 (n=6; P=0.042); LDH: 7575±832 vs. 4729 ±878 (n=6; P=0.040), respectively].

The rate of lipid peroxidation was low in sham-operated mice, as indicated by the low MDA content, which nearly doubled following 60/120 min I/R, and this increase was attenuated in mice pretreated with HU-308, an effect preventable by AM630 pretreatment (Fig. 1C).

An important factor in the tissue damage following I/R is the neutrophil infiltration, an indicator of which is tissue MPO activity. In sham-operated, wild-type mice, MPO activity was barely detectable (Fig. 1D). Sixty/120 min I/R induced a marked increase in MPO activity, which was attenuated by HU-308. AM630 pretreatment attenuated the effect of HU-308.

CB_2 agonist attenuates I/R-induced proinflammatory cytokine and chemokine expression in liver and serum

Sixty/120 min I/R greatly increased the levels of TNF-α, MIP-1α, and MIP-2 in the serum and in liver homogenates, as detected by ELISA (Fig. 2, A and B). HU-308 attenuated the I/R-induced increase significantly in cytokine levels in liver and serum, whereas AM630 pretreatment attenuated these effects of HU-308 (Fig. 2, A and B).

CB_2 agonist attenuates I/R-induced hepatic apoptosis

Sixty/120 min I/R increased caspase 3 activity and DNA fragmentation greatly (Fig. 3, A and B). HU-308 attenuated the I/R-induced, increased caspase 3 activity and DNA fragmentation significantly (Fig. 3, A and B).

CB_2 agonist improves I/R-induced hepatic histopathology

Sham-operated mice showed normal hepatic histology (Fig. 4A). In mice, 24 h following ischemic injury (60 min I), there was a marked degree of reperfusion damage indicated by the necrosis of hepatocytes in the pericentral and midzonal regions and by the massive neutrophil infiltration in the damaged areas (Fig. 4, A and B). The tissue injury and neutrophil infiltration were less pronounced in the HU-308-treated group (Fig. 4, A and B).

CB_2 agonist mitigates TNF-α-induced ICAM-1 and VCAM-1 expression in HLSECs

HLSECs have CB_2 receptors measured by RT-PCR (Fig. 5A). TNF-α (50 ng/ml) treatment of HLSECs for 4 h resulted in robust activation of ICAM-1 (approximately fivefold, Fig. 5, B and C) and VCAM-1 (approximately fourfold, Fig. 5, D and E), respectively, when compared with control. HU-308 (0.5–4 μM) dose-dependently inhibited the TNF-α-induced, increased expression levels of ICAM-1 and VCAM-1 (Fig. 5, B and D), which was attenuated by a CB_2 antagonist (AM630; 1 μM) but not CB_1 antagonists (AM251; 1 3M; Fig. 5, C and E). Antagonists had no effect in controls or TNF-α-treated cells.

CB_2 agonist inhibits TNF-α-induced PMN adhesion to HLSECs

TNF-α (50 ng/ml) treatment resulted in enhanced PMN adhesion to HLSECs (~3.5-fold) compared with control (Fig. 6). HU-308 (4 μM) pretreatment markedly inhibited TNF-α-induced PMN adhesion to HLSECs, and this effect was attenuated by CB_2 antagonists, which had no effect in controls or TNF-α-treated cells on PMN adhesion.

DISCUSSION

There is considerable interest in the development of selective CB_2 receptor agonists, which are devoid of psychoactive properties of CB_1 agonists, for various inflammatory disorders. Earlier studies hypothesized that endocannabinoids or canna-binergic ligands, acting via a CB_1/CB_2-dependent or CB_1/CB_2-independent mechanism, may exert protective effects in various forms of preconditioning (including ischemic) of the myocardium (reviewed in refs. [14,39,41]). However, these studies used buffer-perfused, isolated heart preparations and could not address the question of whether endocannabinoids or synthetic agonists can modulate endothelial or immune cell activation and interactions, which are pivotal events in the sequel of reperfusion damage [14,39]. In a more relevant study by using nonselective CB agonist WIN55212-2 and CB_2 antagonist AM630 in a mouse model of myocardial I/R, the reduction of leukocyte-dependent myocardial damage could be attributed to CB_2 receptor activation, as the protection afforded by WIN55212-2 could be prevented by AM630 but not by CB_1 antagonist AM251 [46]. In agreement with those findings, we have recently reported that CB_2 agonist JWH133 afforded protection against hepatic I/R damage by decreasing inflammatory cell infiltration and consequent tissue injury, and CB_2 receptor knockout mice developed increased injury and proinflammatory phenotype following I/R [44].

In the present study, by using a more selective CB_2 receptor agonist HU-308 [43], we aimed to delineate the role of CB_2 receptor activation in an in vivo model of hepatic I/R and in TNF-α-induced ICAM-1 and VCAM-1 expression in HLSECs and adhesion of human neutrophils to HLSECs in vitro. In addition, we have studied the effect of CB_2 stimulation on I/R-induced oxidative stress and apoptosis. We found that pretreatment of mice with HU-308 decreases the I/R-induced tissue damage, inflammatory cell infiltration, tissue and serum TNF-α, MIP-1α, and MIP-2 levels, tissue lipid peroxidation, and apoptosis. It is important that the beneficial effect of HU-308 was also preserved when injected right before reocclusion. CB_2 receptor was expressed in HLSECs, and its activation attenuated the TNF-α-induced ICAM-1 and VCAM-1 expression and adhesion of human neutrophils to HLSECs in vitro. These results are in agreement with a recent study demonstrating that selective CB_2 agonists (O-3853, O-1966) significantly decrease cerebral infarction and improve motor function after 1 h middle cerebral artery occlusion, followed by 23 h reperfusion in mice, presumable by attenuating the transient-ischemia-induced increase in leukocyte rolling and adhesion to vascular endothelial cells [47]. The role of CB_2 receptors in I/R injury is supported further by increased accumulation of CB_2-positive macrophages derived from resident microglia and/or invading monocytes following cerebral I/R [48]. Several studies have reported increased endocannabinoid levels following cerebral [18,49–52] and hepatic I/R injury [44,53]; however, the role of endocannabinoids in I/R injury remains to be a controversial issue requiring further clarification. One possibility is that endocannabinoids released during I/R may try to limit the hepatic injury by modulating the expression of adhesion molecules and the infiltration and activation of inflammatory cells (mononuclear and polymorpho-nuclear leukocytes are known to express CB_2 receptors [6,21]) by CB_2-dependent mechanisms.

Adhesion molecules mediate the initial attachment of neutrophils to the activated endothelium [54,55], which is a crucial event in initiation of liver I/R injury [55–58]. On reperfusion, TNF-α acts as a continuous stimulator for neutrophil infiltration in the liver, and it also up-regulates the production of chemokines, which may contribute to the up-regulation of cell adhesion molecules and neutrophil activation as well [57]. The increased inflammatory response aggravates oxidative stress further and initiates a vicious cycle, eventually culminating in cell death and organ dysfunction.

In conclusion, our results suggest that CB_2 agonists may protect against hepatic and perhaps other organ I/R injuries by decreasing the endothelial cell activation, the expression of adhesion molecules ICAM-1 and VCAM-1, TNF-α, chemokine (MIP-1α and MIP-2) levels, neutrophil infiltration, lipid peroxidation, and apoptosis. Further studies should also establish the therapeutic window of protection during the reperfusion phase with CB_2 receptor agonists to devise clinically relevant treatment strategies against various forms of I/R. Nevertheless, the beneficial effect of HU-308 observed in our model of I/R coupled with the absence of psychoactive effects of CB_2 stimulation and the recently observed antifibrotic effects of CB_2 receptor in the liver [59] suggest that this approach may represent a novel, promising strategy against hepatic and possibly other forms of I/R injury. Although HU-308 is one of the most selective CB_2 agonists available, as with all drugs, it cannot be excluded entirely that it might have additional beneficial properties not related to CB_2 receptor stimulation.

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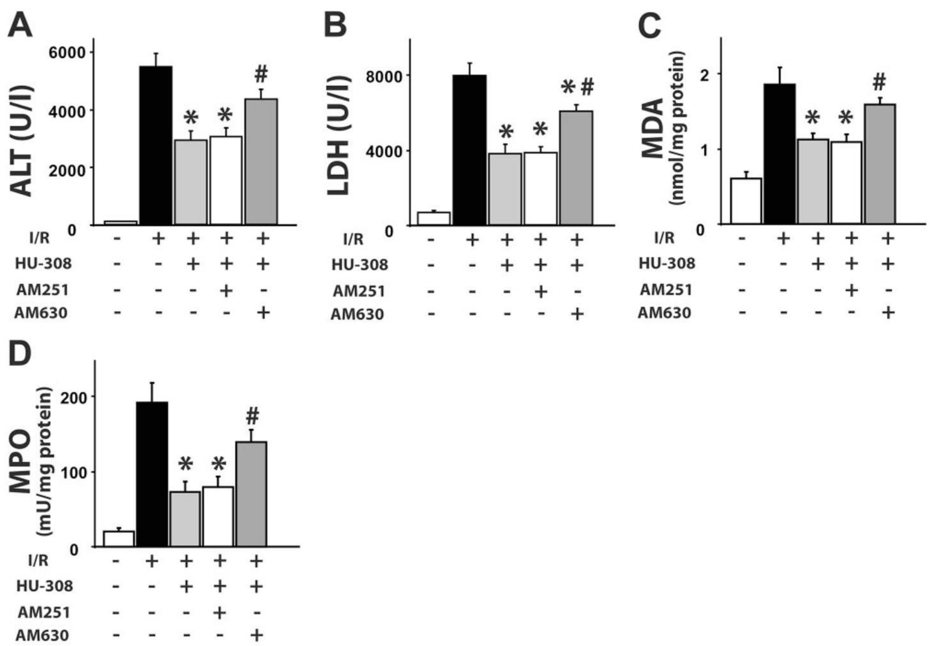

Fig. 1.

CB_2 agonist limits liver I/R-induced damage, lipid peroxidation, and neutrophil infiltration. (A) Serum transaminase ALT and (B) LDH levels; (C) liver MDA level and (D) MPO activity in sham or in mice exposed to 60/120 min I/R, pretreated with vehicle, HU-308 (10 mg/kg), AM251 (1 mg/kg) + HU-308, or AM630 (1 mg/kg) + HU-308 (n=7–12/each group; *, P<0.05, vs. I/R; #, P<0.05, versus I/R+HU-308).

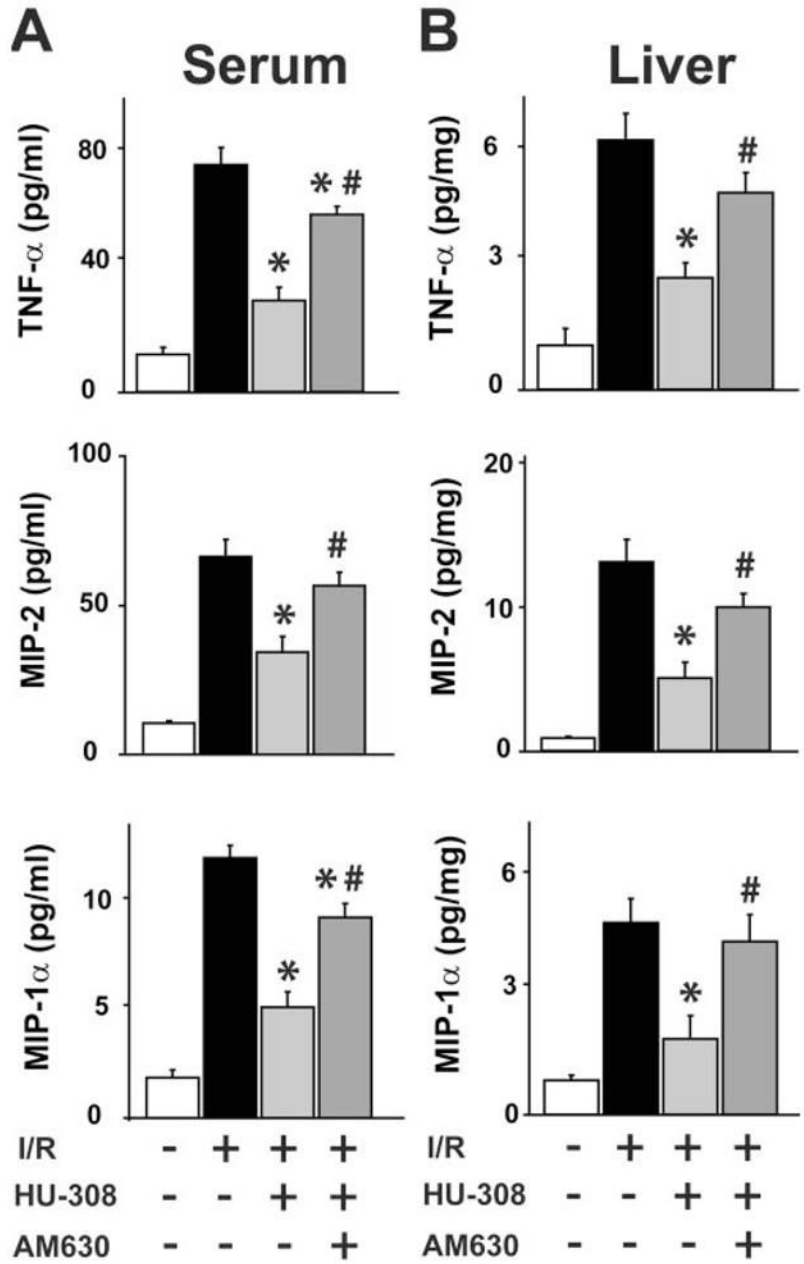

Fig. 2.

CB_2 receptor agonist decreases proinflammatory markers in serum and liver. (A and B) TNF-α, MIP-2, and MIP-1α and levels in serum and liver tissue, measured by ELISA in sham or in mice exposed to 60/120 min I/R, pretreated with vehicle, HU-308 (10 mg/kg), or AM630 (1 mg/kg) + HU-308 (n=6–12/each group; *, P<0.05, vs. I/R; $^{\#}$, P<0.05, vs. I/R+HU-308).

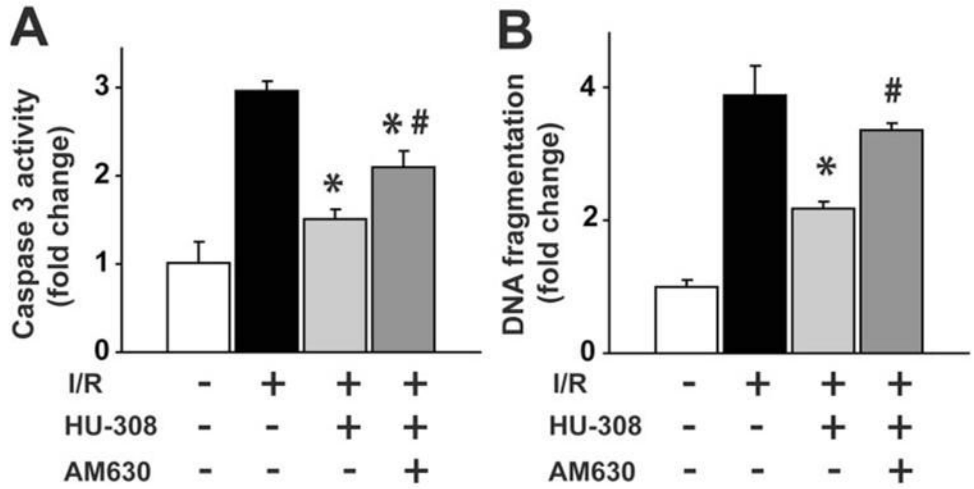

Fig. 3.

CB_2 receptor agonist decreases I/R-induced apoptosis. (A) Caspase 3 activity and (B) DNA fragmentation in sham or in mice exposed to 60/120 min I/R, pretreated with vehicle, HU-308 (10 mg/kg), or AM630 (1 mg/kg) + HU-308 (*n*=6–8/each group; *, $P<0.05$, vs. I/R; #, $P<0.05$, vs. I/R+HU-308).

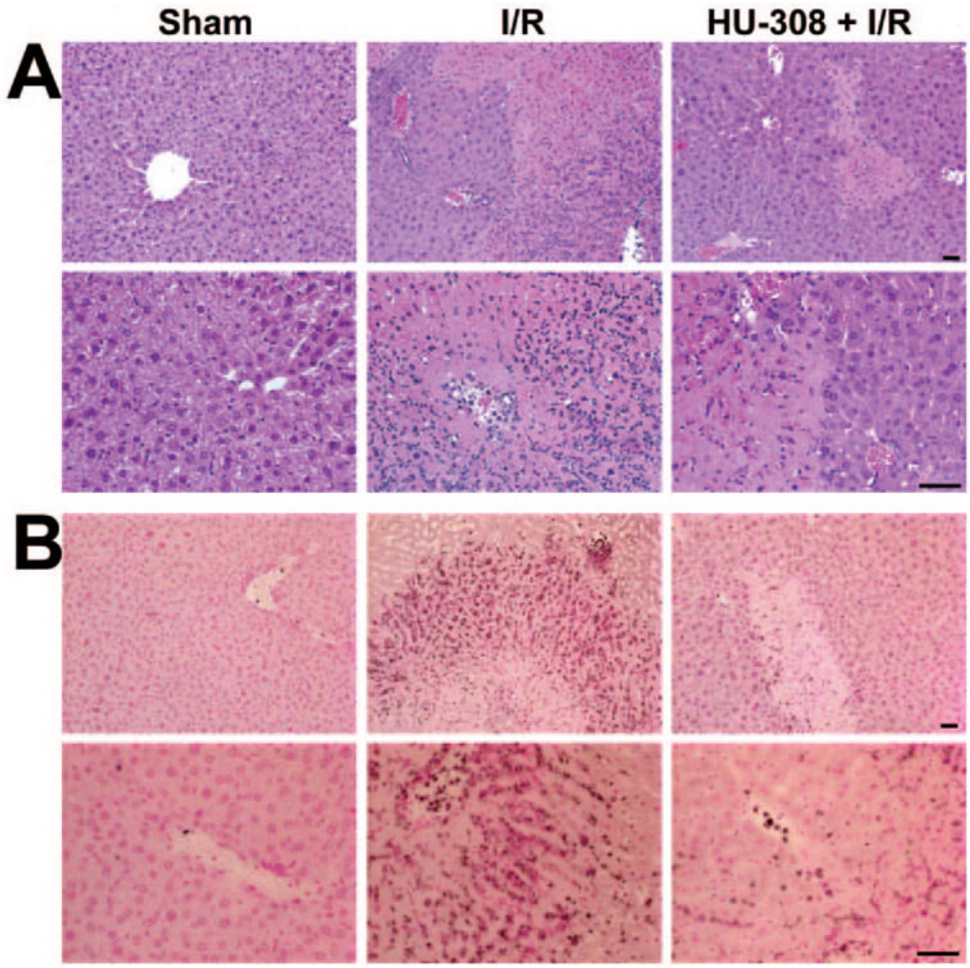

Fig. 4.
CB_2 receptor agonist decreases histological damage and neutrophil infiltration 24 h following ischemia. Representative liver sections of sham mice, of mice exposed to 1 h/24 h I/R with vehicle, or HU-308 pretreatment. (A) H&E staining. (B) MPO staining (brown) contrastained with nuclear fast red. A similar histological profile was seen in three to four livers/group. The original scale bars indicate 50 μm.

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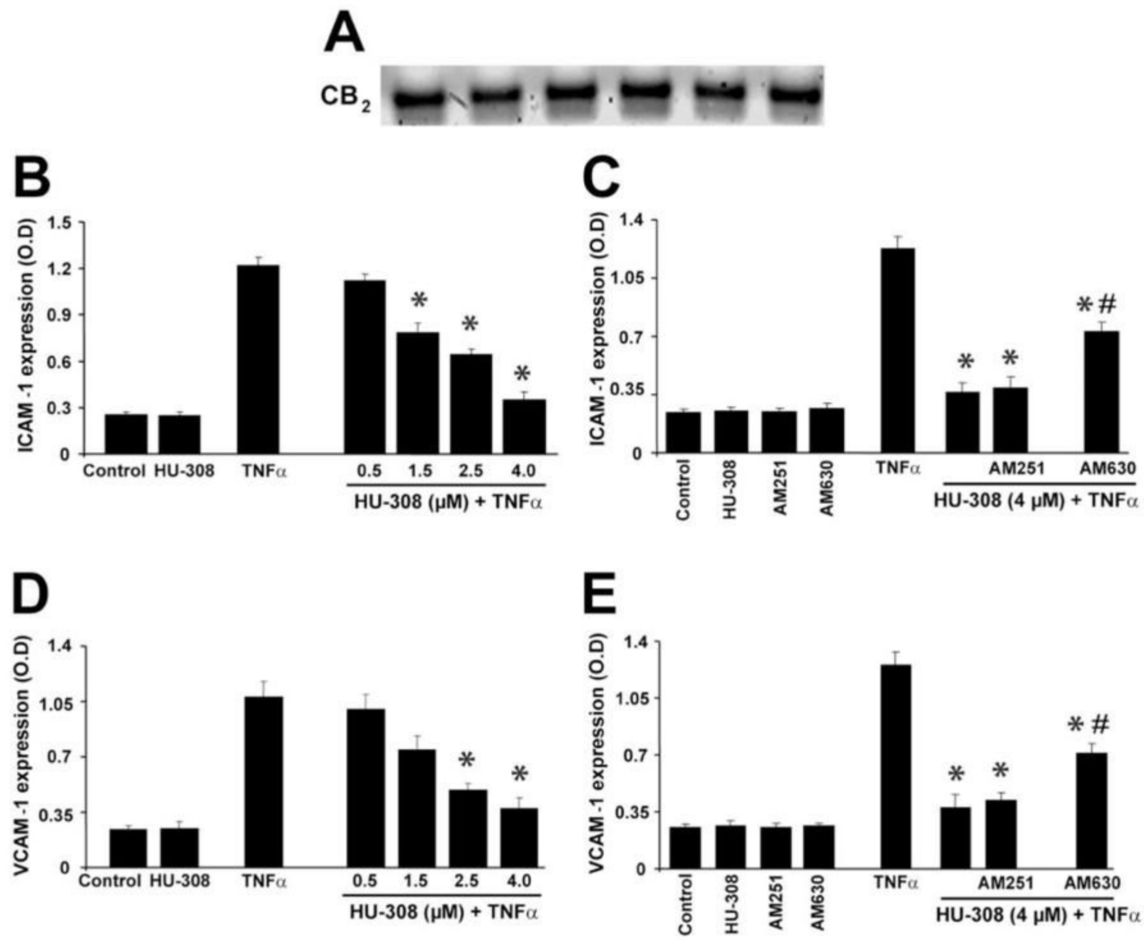

Fig. 5.

CB_2 receptor agonist decreases TNF-α-induced overexpression of ICAM-1 and VCAM-1 in HLSECs. (A) CB_2 receptor expression in HLSECs (RT-PCR). (B and C) ICAM-1 expression; (D and E) VCAM-1 expression (*, P<0.05, vs. TNF-α; #, P<0.05, vs. TNF-α+HU-308; n=9).

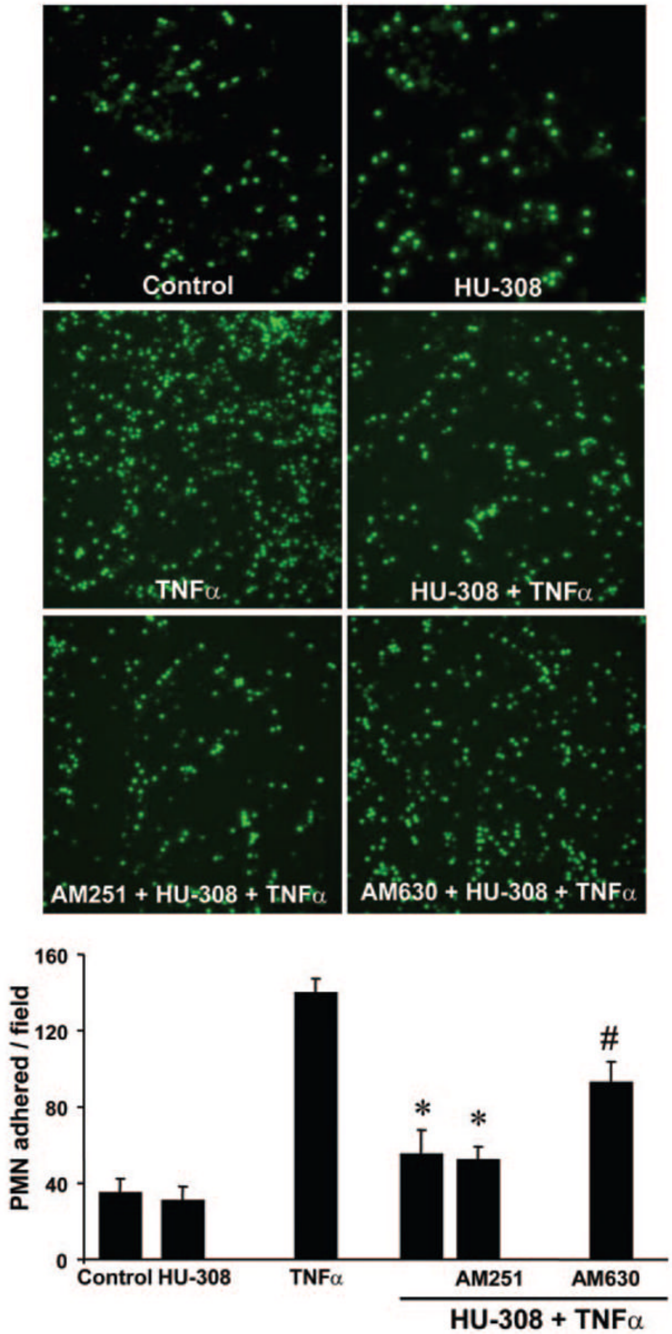

Fig. 6.

CB_2 antagonist attenuates TNF-α-induced neutrophil adhesion to HLSECs. Representative images and quantification of human neutrophil adhesion to human liver endothelial cells. (*, $P<0.05$, vs. TNF-α; #, $P<0.05$, vs. TNF-α + HU-308; $n=4$).