μ -Opioid receptor activation prevents acute hepatic inflammation and cell death

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Background and aims: The detrimental impact of opioid agonist on the clinical management of inflammatory diseases remains elusive. Given the anti-inflammatory properties of the μ -opioid receptor (MOR) agonists at the intestinal barrier, we hypothesised that MOR activation might also dampen acute hepatic inflammation and cell death—major determinants in the pathogenesis of liver diseases.

Patients and methods: The expression of MOR in liver biopsy specimens and peripheral blood mononuclear cells of untreated patients with chronic hepatitis C virus infection and controls, primary hepatocytes and cell lines was determined by quantitative PCR, immunoblotting and/or immunohistochemistry. The effects of peripheral MOR agonist (D-Ala²,NMe-Phe⁴,Gly⁵-ol (DAMGO)) and/or antagonist (naloxone methiodide) were explored in two models of acute hepatitis in mice. MOR-deficient mice were used to evaluate the essential regulatory role of MOR during carbon tetrachloride (CCl₄)-induced hepatitis. The role of DAMGO in cell death was investigated using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) analysis and quantification of lactate dehydrogenase release.

Results: The key role of MOR in the prevention of acute hepatic inflammation and cell death in vivo and in vitro is reported. Whereas MOR gene expression increased transiently in the model of acute liver injury and TNF α -treated HepG2 cells, an impaired expression of MOR mRNA in human chronic hepatitis C samples was found. Furthermore, preventive administration of the selective MOR agonist DAMGO enhanced hepatoprotective-signalling pathways in vivo that were blocked by using naloxone methiodide. Consistently, genetic and pharmacological inhibition of MOR enhanced the severity associated with experimental hepatotoxin-induced hepatitis. Finally, treatment with DAMGO was shown to prevent cell death in vitro in HepG2 cells in a MOR-dependent manner and to prevent concanavalin A- and CCl₄-induced cell death in vivo, providing a possible explanation for the anti-inflammatory role of MOR activation in the liver.

Conclusions: The results indicate that MOR agonists may prevent acute hepatitis and hold promising therapeutic use to maintain remission in both chronic inflammatory bowel and liver diseases.

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Natural opioid peptides, including β -endorphin and endomorphin, are mainly released by immune cells, neurones, the biliary tree and the adrenal glands.^{1,2} A variety of immunocytes, such as natural killer (NK) cells, neutrophils, macrophages, dendritic cells and T and B cells also express opioid receptors (δ , κ and μ).^{3,4} Controlling tolerance/addiction to opiates and immuno-inflammatory processes,⁵ the spatio-temporal induction/repression of opioid receptor expression is controlled by protein degradation, as well as by RNA processing, *cis*-acting functional polymorphisms and multiple transcription factors, such as RXR, NF- κ B, AP-2, SOX and/or Sp1/Sp3.^{6–8} The sensitivity and function of G-protein-coupled opioid receptors are mainly dependent on their expression level and trafficking inside the target cells, as well as on the cellular availability to opioid peptides.

On complex sensing of various potent synthetic or endogenous agonists, the G-protein-coupled opioid receptors function as critical physiological regulators of a broad spectrum of cellular functions. Peripheral ligands of opioid receptors activate mitogen-activated protein kinases and the cAMP-responsive element-binding protein,⁹ resulting in a modulation of intestinal permeability,¹⁰ host defence responses to microbial infection,^{4,11,12} T cell apoptosis,¹³ chemotaxis and inflammatory cytokine production.^{14,15} Notably, μ -opioid receptor (MOR)-deficient mice (MOR^{−/−}) are more susceptible to colitis¹⁶ and an anti-inflammatory function of MOR on explants of inflamed

mucosa from patients with irritable bowel disease has been recently described.¹⁷ Taken together, these observations suggest that endogenous and/or exogenous MOR agonists play a regulatory role in dampening acute host inflammatory reactions. However, the expression and function of MOR in the liver and the physiological relevance of using opioid receptor modulators in the clinical management of liver-associated diseases remain elusive, as the use of opioid antagonists might promote hepatitis C¹⁸ and reduce Fas-induced hepatitis in mice.¹⁹

For reasons still unknown, the plasma content of some natural opioid receptor agonists—namely, met-enkephalins—increases in primary biliary cirrhosis, hepatic encephalopathy, cholestatic pruritus and cholestatic liver diseases.^{20–22} This increased release of opiates might be because of a delayed degradation of endogenous opioids and/or an abnormally induced production of these peptides. Consistently, recent

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; CCl₄, carbon tetrachloride; CNS, central nervous system; ConA, concanavalin A; DAMGO or DO, [D-Ala²,NMe-Phe⁴,Gly⁵-ol]-enkephalin; DOR, Δ -opioid receptor; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; KOR, κ -opioid receptor; LDH, lactate dehydrogenase; MOR, μ -opioid receptor; NF- κ B, nuclear factor κ B; NK, natural killer; NM, naloxone methiodide; TNF α , tumour necrosis factor α ; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

reports indicated a role of opioid antagonists in fibrosis²³ and of endogenous opioid agonists in the course of cholestasis.²⁴ Interestingly, we found impaired expression of MOR in liver samples from patients with chronic hepatitis C, indicating a possible role of MOR in regulating liver injury. Using a biopharmaceutical and genetic approach, we set out to define the role of MOR in two models of acute hepatitis in mice induced, respectively, by concanavalin A (ConA) and carbon tetrachloride (CCl₄). ConA induces a severe hepatitis associated with apoptosis and depending on the activation of T lymphocytes and other leucocytes.²⁵ By contrast, CCl₄ is known to be a hepatotoxin,^{26–28} which causes centrolobular necrosis with acute increases in plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) levels. Here, we describe a new hepatoprotective-signalling pathway dependent on the activation of MOR.

RESULTS

Expression of the μ -opioid receptor in hepatitis C and in hepatocytes

To investigate a potential role of MOR in the liver, we first looked at MOR mRNA expression in human liver samples from controls and patients with hepatitis C.²⁹ Interestingly, we found a significant abnormal MOR gene expression in the liver of patients with hepatitis C compared with controls (fig 1A). This impaired expression was also found in peripheral blood monocytes isolated from patients with hepatitis C when compared with controls (fig 1B). Furthermore, MOR mRNA levels were similar in the whole liver of mice compared with the colon (fig 1C), an organ where the anti-inflammatory roles of MOR have been recently demonstrated.¹⁶ Compared with microdissected human hepatocytes, quantitative analysis of MOR gene expression showed significantly lower amounts of MOR mRNA in the whole liver, suggesting that hepatocytes are a major hepatic source for this receptor (fig 1C). Accordingly, we showed similar expression levels of MOR mRNA between two human hepatoma cell lines (ie, HepG2 and HuH7) and two cell lines known to express MOR (ie, the human intestinal epithelial cell line HT-29 and the human monocytic cell line THP1; fig 1D).^{30–31} Finally, we specifically detected MOR protein in the HepG2 cell line (fig 1E). Taken together, these results indicate that MOR mRNA and protein are expressed in hepatocytes, providing a basis to begin to understand the function and regulation of this receptor in this barrier organ.

Increased MOR expression by pro-inflammatory cytokines

Before evaluating the potential therapeutic efficacy of MOR activation during hepatic inflammation, we compared the physiological levels of MOR expression in the healthy and inflamed liver of mice and investigated whether a treatment with tumour necrosis factor- α (TNF) might increase its expression *in vitro*.

To characterise the regulation of MOR expression *in vivo*, we used the ConA-induced inflammation model of hepatitis, which is characterised by the production of inflammatory mediators in the liver, such as TNF α , interleukin (IL) 1 β and interferon (IFN) γ , and by a cytolysis of hepatocytes (fig 2A). In this model, hepatic levels of MOR mRNA paralleled the induced expression of TNF α , IL1 β and INF γ mRNA (fig 2A). However, we failed to detect any change in hepatic MOR protein level by specific immunoblotting after ConA administration (fig 2B).

To get further insights into the regulation of MOR expression in this organ, we next analysed the effect of TNF- α on expression of MOR mRNA in HepG2 cell line. TNF- α treatment significantly enhanced MOR mRNA expression in a dose- and time-dependent manner (fig 2C,D; $p < 0.05$). Taken together,

induction in the liver of MOR expression by hepatocytes during inflammation supports the notion that MOR activation might be an attractive therapeutic target to modulate acute hepatic inflammation.

MOR activation prevents CCl₄- and ConA-induced hepatitis in mice

To evaluate the physiological therapeutic effect of MOR agonists during experimental hepatitis in mice, we compared biochemical and histological markers of liver injury in mice treated or not with a specific and peripheral MOR agonist DAMGO (hereafter DO),^{32–33} given preventively at the optimal dosage of 10⁻³ mg/kg/day.¹⁶ As expected, CCl₄ administration in Balb/c mice induced a predominant pericentrolobular necrosis reaching up to 50% of the lobular surface in 24 h (fig 3A,B) and a significant increase in the plasma AST, ALT and lactate dehydrogenase (LDH) levels (fig 3C–E). By contrast, DO-treated mice showed a significant reduction of the necrotic area (fig 3A,B), together with a marked decrease of serum ALT and LDH release (fig 3C–E). Conversely, systemic administration of a peripheral antagonist of opioids—namely, naloxone methiodide (NM, also referred to as methylnaltrexone) significantly attenuated the effect of DO during the course of CCl₄-induced hepatitis (fig 3). Consistently, exposure to DO significantly reduced the CCl₄-induced recruitment of CD25, CD8a and CD49b inflammatory cells (fig 3F–H). Furthermore, preventive administration of DO improved significantly the ConA-induced liver injury compared with control mice, as reflected by a significant decrease in serum AST, ALT and LDH levels (fig 4A–C) and production of inflammatory cytokines, such as TNF α , IL1 β and IFN γ (fig 4D–F). Taken together, our results shed light on the anti-inflammatory properties of the MOR agonist in the liver.

MOR-dependent regulation of hepatoprotection *in vivo*

Given the regulatory effect of DO treatment in liver inflammation, we performed a pharmacological inhibition of peripheral MOR in mice with healthy livers and in mice with CCl₄-induced hepatitis. Whereas treatment with NM alone did not modify plasma levels of liver enzymes nor hepatic histological evaluation, liver cytolysis and histological lesions of necrosis were significantly more pronounced in NM-treated mice with CCl₄-induced hepatitis compared with control animals (fig 5A–D), indicating that endogenous opioids control the severity of the CCl₄-induced lesions.

To assess the essential role of MOR in our model of liver injury, we next investigated whether (MOR^{-/-}) mice³⁴ were more susceptible to CCl₄-induced hepatitis. No significant differences in plasma levels of liver enzymes, morphological and histological liver analysis in untreated mutant and wild-type mice were observed (fig 5E–H). In contrast, 24 h after CCl₄ administration, cytolysis evaluated by plasma dosages of AST (MOR^{-/-} 7314 (1540) vs MOR^{+/+} 5243 (1035 IU), ALT (MOR^{-/-} 10 112 (2480) vs MOR^{+/+} 6264 (1340 IU) and LDH (MOR^{-/-} 36 510 (7872) vs MOR^{+/+} 16 070 (5350 IU)), revealed that the degree of liver inflammation and cell death was higher in MOR^{-/-} mice than in their wild-type littermates ($p < 0.05$; fig 5E–H). To evaluate whether another defective expression of opioid receptor may also affect the susceptibility of mice to liver inflammation, we induced hepatitis using CCl₄ in Δ -opioid receptor (DOR) knockout mice (DOR^{-/-}).³⁵ Markers of liver inflammation including plasma dosages of AST, ALT and LDH and surface of necrosis area were similar in DOR^{-/-} and control mice (fig 5E–H) suggesting that MOR, but not DOR, acts as a protective G-protein-coupled opioid receptor against CCl₄-induced liver injury in mice. Taken together, our results

Figure 1 Impaired expression of μ -opioid receptor (MOR) in patients with chronic hepatitis C and MOR expression in hepatocytes. (A) Levels of MOR mRNA in liver biopsy specimens of untreated controls (open circles) and patients with chronic hepatitis C (closed circles) in two different cohorts.²⁹ Median (SEM) is represented. (B) Levels of MOR mRNA in peripheral blood monocytes isolated from controls (white squares, $n = 17$) and patients with hepatitis C (black squares, $n = 14$). Median (SEM) is represented. (C) Levels of MOR mRNA in the normal whole brain, liver, colon and microdissected human hepatocytes. Values obtained from four independent experiments represent the relative mean (SEM) of triplicate data. (D) Levels of MOR mRNA in human epithelial cells (HT-29), hepatocytes (HepG2 and HuH7), monocytes (THP1) and lymphocytes (Jurkat). Values obtained from four independent experiments represent the relative mean (SEM) of triplicate data. (E) MOR-stained HepG2 cells with a membranous localisation of the receptors (magnification $\times 100$). Insert: Absence of staining in negative control corresponding to the use of an irrelevant antibody. p Value was determined by the Mann-Whitney U test. HCV, hepatitis C virus.

indicate that MOR has essential anti-inflammatory properties in the liver as previously shown in the colon.

MOR activation prevents cell death in vivo and in vitro

To obtain further insights into the regulatory role of MOR during hepatitis, we determined the impact of DO on cell death in two models of hepatitis. Consistent with our initial data on LDH

release, we showed a significant decrease in CCL₄- and ConA-induced apoptosis following the administration of DO by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) analysis (fig 6A,B; $p < 0.05$), suggesting an anti-apoptotic effect of MOR activation by DO in the liver. Therefore, we assessed the impact of DO on ceramide-induced cell death in HepG2 cells by quantifying the release of LDH. We found a

Figure 2 Regulation of μ -opioid receptor (MOR) expression in hepatocytes by inflammation. (A) Kinetics of plasma hepatic enzymes (ALT, AST, LDH) and liver mRNA expression of MOR and cytokines (IFN γ , TNF α , IL1 β) at 1 ($n = 10$) and 8 h ($n = 10$) after concanavalin A (ConA) administration in mice. Values obtained from two independent experiments represent the mean (SEM) expressed as fold induction compared with untreated animals ($n = 10$). (B) MOR immunoblotting analysis in liver at 0 ($n = 3$), 1 ($n = 3$) and 8 h ($n = 3$) after ConA administration in mice. Ratio between signals of MOR and β -actin immunoblotting is shown. (C) Dose-dependent effect of TNF α on MOR mRNA expression in HepG2 cells treated with 10, 50, 100 ng/ml for 6 h. Values obtained from two independent experiments represent the mean (SEM) of triplicate data expressed as fold induction compared with untreated HepG2 cells. (D) Time-dependent effect (3, 6, 9, 24) of TNF α (100 ng/ml) on MOR and cytokines (IFN γ , TNF α , IL1 β) mRNA expression in HepG2 cells. Values obtained from two independent experiments represent the mean (SEM) of triplicate data expressed as fold induction compared with untreated HepG2 cells. p Value was determined by the Mann-Whitney U test. ALT, alanine transaminase; AST, aspartate aminotransferase; IL, interleukin; IFN, interferon; LDH, lactate dehydrogenase; TNF, tumour necrosis factor.

Figure 3 Anti-inflammatory role of μ -opioid receptor (MOR) agonist in mice with carbon tetrachloride (CCL₄)-induced hepatitis. Representative histological sections of liver of mice treated or not with [D-Ala², NMe-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO; denoted as DO), naloxone methiodide (NM) and/or CCL₄ (A); graphical representation of liver necrosis (B); and plasma enzyme levels of AST, ALT and LDH in Balb/c mice ($n = 10$ per group) (C-E); relative mRNA expression of CD25, CD8 α and CD49b in the liver of Balb/C mice ($n = 10$ per group) (F-H). Values represent the mean (SEM). p Value was determined by the Mann-Whitney U test. AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

significant dose-dependent decrease in ceramide-induced cell death, which was blocked by incubating with the specific and peripheral MOR antagonist NM (fig 6C). Consistently, we also found a significant decrease in serum withdrawal-induced apoptosis after treatment with DO on HepG2 cells by TUNEL analysis (data not shown). Taken together, we showed anti-apoptotic properties of MOR in hepatocytes.

MATERIALS AND METHODS

Reagents

The MOR agonist DO,³⁶ the general opioid antagonist NM, ConA, CCL₄ and recombinant human TNF α were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

Liver biopsy specimens and peripheral blood mononuclear cells

Liver biopsies specimens used in the present work were described previously.²⁹ Peripheral blood mononuclear cells were separated on Ficoll gradient (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) and washed in PBS until analysis of MOR by real-time PCR and immunohistochemistry. Peripheral blood was collected from 17 control patients and 14 patients with chronic hepatitis C virus infection.

Cell lines

Human hepatoma cell line HepG2 (ATCC HB 8075), HuH7 (ATCC CCL-185), colon carcinoma cell line HT-29 STD (ATCC HTB-38), monocytic cell line THP-1 (ATCC TIB 202) and human T cell line Jurkat (ATCC TIB 152) were obtained from the American Type Culture Collection. Cell lines were grown at 37°C in Dulbecco's modified Eagle medium (Sigma-Aldrich, USA) or RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Eurobio, Paris France), in a humidified atmosphere of 5% CO₂ and 95% relative humidity.

To evaluate the regulation of MOR expression by inflammatory cytokines in hepatocytes, HepG2 cells were stimulated for 3, 6, 9 and 24 h with 10, 50 and 100 ng/ml of recombinant TNF α . All experiments were performed in triplicate and repeated three times for confirmation. Cell viability was determined by trypan blue exclusion.

For microdissected hepatocytes, 8 μ m RNase-free frozen sections were cut with a standard cryostat at -20°C, mounted on a polyethylene glass slide and stained with May-Grunwald Giemsa. Hepatocytes were then microdissected using the Leica Laser Microdissection system (Leica Microsystems, Wetzlar, Germany) and collected in 0.5 ml RNase-free sterile tubes.

Figure 4 Beneficial role of μ -opioid receptor (MOR) agonist in mice with cononavalin A (ConA)-induced hepatitis. After 8 h of treatment with or without ConA, plasma enzyme levels (A, B), lactate dehydrogenase (LDH) (C) and liver cytokine mRNA concentrations (D–E) in Balb/c mice treated (n = 5) or not (n = 5) with the selective MOR agonist [D-Ala², NMe-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO; denoted as DO) were compared with control animals (n = 5). Values represent the mean (SEM). p Value was determined by the Mann–Whitney U test. ALT, alanine transaminase; AST, aspartate aminotransferase; IFN, interferon; TNF, tumour necrosis factor.

Figure 5 Deleterious effects of μ -opioid receptor antagonist and susceptibility of MOR^{-/-} mice to carbon tetrachloride (CCL₄)-induced hepatitis. Plasma enzyme levels (A–C) and representative liver abnormalities (D) in Balb/c mice with CCL₄-induced hepatitis receiving the opioid antagonist naloxone methiodide (NM) (black square, n = 10) or not (white square, n = 10) compared with control animals (n = 10). Values represent the mean (SEM). p Value was determined by the Mann–Whitney U test. A representative percentage of the necrotic area in the liver is indicated. Plasma enzyme levels (E–G) and representative liver abnormalities (H) in MOR^{-/-} (black square, n = 13) and DOR^{-/-} (grey square, n = 8) mice with CCL₄-induced hepatitis and compared with control age and sex-matched animals (n = 5). Values represent the mean (SEM). p Value was determined by the Mann–Whitney U test. A representative percentage of the necrotic area in the liver is indicated. ALT, alanine transaminase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

Figure 6 [D-Ala²,NMe-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) treatment reduces cell death in vivo and in vitro. (A) terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL)-positive cells were counted from three independent fields (magnification $\times 10$) of liver sections from each mouse treated or not with concanavalin A (ConA) and/or DAMGO ($n=3$). Values represent the mean (SEM) of triplicate raw data. (B) TUNEL-positive cells were counted from three independent fields (magnification $\times 10$) of liver sections from each mouse treated or not with CCL₄ and/or DAMGO ($n=3$). Results are expressed as the mean (SEM). (C) Analysis of cell death by measuring lactate dehydrogenase release by HepG2 cells. The concentrations of DAMGO and naloxone methiodide (NM) used are indicated in μ M. Ceramide was used at a concentration of 100 μ M.

For analysis of cell death in the HepG2 cell line, cells were seeded at 5×10^4 /sterile coverslip in a 96-well plate and cultured overnight in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum. HepG2 cells were treated with DO at 0.01–10 μ M in serum culturing media for 18 h. The release of lactate dehydrogenase was measured in the collected supernatants. For TUNEL analysis in the HepG2 cell line, cells were seeded at 2×10^5 /sterile coverslip in a 24-well plate and cultured overnight in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum. HepG2 cells were treated with DO at 10^{-4} and 10^{-5} g/ml in serum-withdrawn culturing media for 18 h. The experiment was performed in triplicate and repeated twice for confirmation.

Semi-quantitative and real-time quantitative RT-PCR

Total RNA was isolated from cells and tissue samples using the Nucleospin RNA II Kit (Macherey Nagel, France) according to the manufacturer's instructions. RNA was quantified using spectrophotometry. After treatment at 37°C for 30 min with 20–50 RNase-free DNase I, oligo-dT primers were used to synthesise single-stranded cDNA (Roche Diagnostics, Indianapolis, Indiana, USA). Real-time PCR in triplicate was performed using SYBR green Master Mix with specific human and mouse oligonucleotides in a GeneAmp Abiprism 7000 (Applied Biosystems, France). SYBR green dye intensity was analysed using the Abiprism 7000 SDS software (Applied Biosystems, France). All results were normalised to the human or mouse gene β -actin (table 1). The sizes of human and mice PCR products (Accession number AY521028) were tested on an agarose gel with ethidium bromide staining on agarose (3%) and sequenced on the Applied Biosystems model 377 DNA sequencers (Applied Biosystems, Foster City, California, USA).

Immunohistochemistry of MOR in HepG2 cell line

HepG2 cells (10^5 cells/slide) were fixed in fresh 4% paraformaldehyde/PBS, washed in PBS and pre-incubated in 3% H₂O₂ methanol for 20 min. To block non-specific binding, sections were incubated for 1 h with 0.1% bovine serum albumin and then incubated overnight at 4°C with the primary rabbit polyclonal antibody directed against MOR (dilution 1:500, Diasorin 24216, Stillwater, Oklahoma, USA). After a wash with PBS, sections were incubated for 1 h with a secondary biotinylated anti-rabbit IgG antibody (dilution 1:1000, Dako A/S Denmark, Glostrup, Denmark) and for 30 min with biotin-peroxidase-labelled streptavidin (ABCComplex/HRP, Dako A/S

Denmark). Immunocytochemistry was revealed by adding the peroxidase substrate 3,3'-diaminobenzidine (Liquid DAB Substrate-Chromogen System, Dako A/S Denmark). Cells were counterstained with haematoxylin. Negative controls consisted of omission of the first antibody and the use of normal rabbit immunoglobulins (Dako, Paris France).

Induction of CCL₄- and ConA-induced hepatitis in mice

Animal experiments were performed in accredited establishments at the Institut Pasteur, Lille, France according to the governmental guidelines number ° 68/609/CEE. Animals were housed ten per cage and had free access to standard mouse chow and tap water. For hepatitis induction, 7-week-old male Balb/c mice (20–25 g) were anaesthetised and received either an intraperitoneal injection of CCL₄ (300 mg/ml in a volume of 100 μ l) or a retro-orbital injection of ConA (30 mg/kg in a volume of 200 μ l of PBS).²⁵ Control mice received a retro-orbital or an intraperitoneal injection of 200 μ l of PBS or 100 μ l of olive oil, respectively. Animals were euthanised 1 and 8 h after ConA administration or 24 h after CCL₄ injection.

To evaluate the anti-inflammatory effect of MOR activation in vivo, we used the selective agonist DO that lacks the ability to cross the blood–brain barrier.^{32–33} DO was administered once daily by subcutaneous injection of the optimal dose of 10^{-3} mg/kg/day, starting 4 days before hepatitis injection.¹⁶ When mentioned, NM was administered once daily for 5 days before hepatitis induction by subcutaneous injection at a dose of 2.5 mg/kg/day.^{16–37}

To evaluate the critical regulation of liver inflammation through MOR, CCL₄-induced hepatitis was performed as described above in C57BL/6J MOR^{-/-}, C57BL/6J DOR^{-/-} and C57BL/6J wild-type mice (22–24 weeks of age) at the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France. Furthermore, the MOR antagonist NM, which does not cross the blood–brain barrier,³⁸ was given in C57BL/6J wild-type mice preventively and concomitantly 5 days before CCL₄-induced hepatitis induction at the dose of 2.5 mg/kg/day as described previously.³⁷ Samples of the liver and colon were taken from control mice and animals with CCL₄- and ConA-induced hepatitis, immediately frozen or fixed in fresh 4% paraformaldehyde/PBS.

Histological and biological assessment of hepatitis in mice

To assess the severity of the hepatitis, we quantified the plasma level of ALT and AST at 24 h, and 1 and 8 h after CCL₄ or ConA

Table 1 Primer sequences used for real-time PCR

Primer name	Primer sequence (5'-3')
Mouse β -Actin For	TGG AAT CCT GTG GCA TCC ATG AAA C
Mouse β -Actin Rev	TAA AAC GCA GCT CAG TAA CAG TCC G
Human β -Actin For	AAA TCT GGC ACC ACA CCT TC
Human β -Actin Rev	GGG GTG TTG AAG GTC TCA AA
Mouse MOR For	TAT GCG TTC CTG GAT GAA AAC
Mouse MOR Rev	GGT GTG CCT GGT GGT TAG TT
Human MOR For	GAT CAT GGC CCT CTA CTC CA
Human MOR Rev	TGG TGG CAG TCT TCA TCT TG
Mouse TNF α For	TGG GAG TAG ACA AGG TAC AAC CC
Mouse TNF α Rev	CAT CTT CTC AAA ATT CGA GTG ACA A
Human TNF α For	TCA GCC TCT TCT CCT TCC TG
Human TNF α Rev	GCC AGA GGG CTG ATT AGA GA
Mouse IFN γ For	TGG CTC TGC AGG ATT TTC ATG
Mouse IFN γ Rev	TCA AGT GGC ATA GAT GTG GAA GAA
Human IFN γ For	GCA GAG CCA AAT TGT CTC CT
Human IFN γ Rev	ATG CTC TTC GAC CTC GAA AC
Mouse IL-1 β For	TTG ACG GAC CCC AAA AGA TG
Mouse IL-1 β Rev	AGA AGG TGC TCA TGT CCT CAT
Human IL-1 β For	GGA TATGGA GCA ACA AGT GG
Human IL-1 β Rev	ATG TAC CAG TTG GGG AAC TG

IFN, interferon; IL, interleukin; MOR, μ -opioid receptor; TNF, tumour necrosis factor.

administration, respectively. Excised liver specimens were fixed in 4% paraformaldehyde/PBS or kept frozen for further histological examination, TUNEL labelling, immunoblotting and real time reverse transcriptase PCR analysis. Liver protein extraction and immunoblotting were performed as described previously.²⁹ NIH Image 1.61 (<http://www.tsc.undel.edu/macsoftdist/image.html>) was used to calculate the local area of liver necrosis and the ratio between signals of MOR and β -actin immunoblotting from video micrograph tiff files. The mean area of injury with standard deviation was calculated as an average percentage for the most injured field (magnification $\times$ 2.5) from each liver for each study group.

Statistics

Data were expressed as mean (SEM). Statistical analyses were performed using the StatView 4.5 statistical program. All comparisons were analysed by the non-parametric Kruskal–Wallis one-way analysis of variance test or when needed by the Mann–Whitney U and Student's t test. Differences were considered significant when the p value was <0.05.

DISCUSSION

Widely distributed within the central nervous system (CNS), MORs, DORs and k-opioid receptors (KORs) regulate several physiological functions (ie, inflammation and pain) outside the CNS, such as in the intestine. However, the distribution and the regulatory role of opioid receptors in immuno-inflammatory responses in peripheral tissues and particularly in the liver remain confusing.^{19–39} In the present study, the healthy liver of mice, as well as microdissected human hepatocytes and the human hepatocellular carcinoma cell lines HepG2 and HuH7, express MOR mRNA at a level similar to the colon, a peripheral tissue responsive to μ -opioid agonists.¹⁶ Furthermore, we detected consistent MOR protein expression at the surface of HepG2 cells, suggesting a possible regulatory role of MOR in the liver.

Given the role of opioid receptors in the pathogenesis of hepatitis C virus infection,^{18–40–41} and because functions of opioid receptors depend on the level of receptor expression,⁴² we further investigated whether chronic hepatitis C virus infection and inflammatory mechanisms might regulate MOR expression in the liver and/or in hepatocytes. Interestingly, we found an impaired expression in liver biopsy specimens and peripheral

blood mononuclear cells of patients with chronic hepatitis C virus infection. This abnormal expression of MOR might result through inhibition of innate immune signalling pathways during HCV replication. Alternatively, recent reports identified potential untranslated polymorphisms that might control MOR gene expression.^{43–45} In comparison with the normal liver, a two- to sixfold increase of MOR mRNA, but not protein, expression was observed in vivo in the validated model of ConA-induced hepatitis in mice. A similar increase in MOR gene expression was observed in vitro in TNF α -stimulated HepG2 cells, which might be dependent on NF- κ B binding sites located in the MOR gene promoter.^{6–7–46–49} Consistently, inflamed colon^{16–17–50} and rat paw⁸ also showed an increased expression of MOR. Further work is now required to dissect the *cis*- and *trans*-regulation of MOR gene expression in the liver.

The physiological synthesis of endogenous opioid agonists in the liver in health and disease^{50–53} led us to investigate the role of MOR activation during acute hepatitis. Using a genetic and pharmacological approach using two different experimental models of hepatitis induced by CCl₄ or ConA administration, we found MOR playing an essential role in regulating liver inflammation and having a general anti-inflammatory effect of DO, a selective agonist of peripheral MOR.^{32–33} These results suggest a tonic action of MOR in the protection against hepatic inflammation. However, despite a proposed hepatic expression of DOR and the affinity of DOR to opioids found in the liver such as met-enkephalins,^{39–54} the intensity of CCl₄-induced hepatitis was similar in DOR^{−/−} mice compared with controls. Furthermore, given the absence of KORs in the liver,^{19–39} these results might suggest a major role of MOR in hepatoprotection. The role of DOR in additional experimental and natural models of hepatitis will need further attention. Finally, our work supports the notion that DO prevents cell death^{55–57} and suggests that opioid agonists in the liver might function through direct stimulation of hepatocytes and/or in cooperation with immunocytes and/or the sympathetic nervous system. Further work should now clarify the potential role of other cell types in the anti-inflammatory properties of DO in the liver, and the potential anti-fibrogenic properties of MOR in several chronic models of hepatitis.

In conclusion, our data demonstrate a responsiveness of hepatocytes to a specific MOR agonist and a hepatoprotective role of this opioid receptor during hepatitis, suggesting a potential of hepatocytes in controlling pain, fibrosis and/or inflammation of the liver. The underlying anti-inflammatory and anti-apoptotic properties, as well as the nature of the different adaptors of the MOR-signalling pathway, in the liver needs further attention. Our results also suggest that treatment with an opioid receptor antagonist might be detrimental to patients with abnormal acute hepatic function. In summary, development of specific MOR agonists targeting the liver, but not the CNS, holds promising therapeutic opportunities to maintain remission in chronic inflammatory digestive diseases by limiting inflammation and visceral pain.

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