doi:10.1111/j.1440-1746.2006.04260.x

HEPATOLOGY

Opioid system blockade decreases collagenase activity and improves liver injury in a rat model of cholestasis

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Key words
cholestasis, collagenase, endogenous opioids, liver injury, matrix metalloproteinase, nitric oxide, S-adenosylhomocysteine, S-adenosylmethionine.

Accepted for publication 23 October 2005.

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Abstract

Background: Following bile duct ligation (BDL) endogenous opioids accumulate in plasma and play a role in the pathophysiology and manifestation of cholestasis. Evidence of centrally mediated induction of liver injury by exogenous opioid agonist administration, prompts the question of whether opioid receptor blockade by naltrexone can affect cholestasis-induced liver injury.

Methods: Cholestasis was induced by BDL and cholestatic and sham-operated rats received either naltrexone or saline for 7 consecutive days. On the 7th day, liver samples were collected for determining matrix metalloproteinase-2 (MMP-2) activity, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) content and blood samples were obtained for measuring plasma nitrite/nitrate and liver enzyme activities.

Results: Naltrexone-treated BDL animals had a significant reduction in plasma enzyme activity and nitrite/nitrate level. Liver SAM : SAH ratio and SAM level improved by naltrexone treatment in cholestatic animals compared to saline-treated BDL ones. Naltrexone treatment in BDL rats led to a decrease in the level of liver MMP-2 activity, which had already increased during cholestasis.

Conclusion: Opioid receptor blockade improved the degree of liver injury in cholestasis, as assessed by plasma enzyme and liver MMP-2 activities. The beneficial effect of naltrexone may be due to its ability to increase liver SAM level and restore the SAM : SAH ratio.

Introduction

Cholestasis causes hepatocellular injury and leads to progressive hepatic fibrogenesis. Identification of the signals that upset the maintenance of proper liver function and regeneration, or trigger necrosis, apoptosis and fibrogenesis is extremely valuable in understanding the causes of liver damage and in working towards its treatment.

Matrix metalloproteinases (MMP), a family of calcium–zinc-dependent endopeptidases, regulate hepatic remodeling and its response to injurious stimulus.[1] They are secreted as proenzymes and then undergo cleavage to form the active enzyme. Matrix metalloproteinase-2 (72-kDa type IV collagenase, or gelatinase A) is secreted by hepatic stellate cells (HSC) and has been found to degrade type IV collagen.[2] In normal liver, type IV collagen is an important component of the normal matrix and exerts major effects on the morphology and cell-specific function of hepatocytes; hence, its degradation correlates with clinically manifested liver injury.[2,3] Disruption of the normal hepatic matrix hastens its replacement by scar matrix, which will lead to hepatic dysfunction.

The amount of expression and activity of MMP are influenced by diverse factors. One of the important factors is hypomethylation of their genes that enhances the expression of MMP.[4,5] Other factors such as nitric oxide (NO) and other free radicals can also contribute to the level of expression and activity of MMP.[6,7]

Many physiological and behavioral functions are under the influence of the opioid system. Alteration in the opioid system has been reported in patients with liver disease.[8] Increased opioid neuromodulation and elevated plasma level and activity of opioid peptides have been demonstrated in humans and animals subjected to cholestasis.[9,10] It is believed that the activation of the opioid system may contribute to the pathophysiology of cholestatic liver disease.[10–13]

Opioid peptides (endorphins, enkephalins, and dynorphins) act through three classes of receptors: μ, κ, and δ opioid receptors, all

of which are antagonized by naltrexone, a long-acting opioid receptor blocker. Acute and chronic activation of opioid receptors in animals have been shown to induce liver damage as assessed by increased oxidative stress and plasma liver enzyme activities.[14–16] Liver injury has also been induced by intracerebroventricular (i.c.v) administration of small amounts of opioid agonists, suggesting a possible interaction of brain and liver for the deleterious effects of opioids on hepatocytes.[15,16]

The increased opioidergic tone in cholestasis and the possibility of its contribution to liver damage led us to investigate whether chronic opioid receptor blockade can influence liver injury in short-term bile duct-ligated (BDL) rats. We also tried to determine some of the responsible mechanisms.

Methods

Reagents

All materials were purchased from Sigma Aldrich (Poole, Dorset, UK) unless otherwise stated.

Animals

Adult male Sprague–Dawley rats (215–225 g, Pasteur Institute of Iran, Tehran, Iran) were used throughout the present study. The animals were housed in a temperature-controlled room (23 ± 1°C), on a 12-h regular light–dark cycle with free access to standard laboratory rat chow and water. They were handled in accordance with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (NIH US publication no. 85–23 revised 1985). All experiments were performed in line with the ethical considerations, recommended by the Pasteur Institute of Iran.

The rats were randomly divided into four groups, each consisting of age- and weight-matched rats ($n = 8$ in each group at the time of sampling). Two groups were sham operated (control groups) and the other two groups underwent BDL.[17] Briefly, after midline laparotomy under general anesthesia,[11–13] the common bile duct was exposed. In BDL rats, the bile duct was double ligated with silk then sectioned between two ligatures; whereas in the sham-operated rats the bile duct was manipulated and no ligation or resection was performed. Finally the abdominal wall was closed in two layers.

Drug administration and sample collection

One group of sham-operated and BDL rats were treated with daily s.c. administration of isotonic sterile saline solution (1 mL/kg per day). The second group of sham-operated and BDL rats received daily s.c. injection of naltrexone (20 mg/kg per day;[12,13] Iran Daru, Tehran, Iran) for 7 consecutive days. Seven days after cholestasis, the animals were killed under sodium pentobarbital anesthesia (50 mg/kg; i.p.). Liver samples were collected and blood samples obtained via cardiac puncture.

We used 20 mg/kg per day of the drug, which has been used many times for opioid system blockade in rats, according to the literature.[11,18] This dose could also prevent the cardiovascular manifestation of opioid system in cholestasis, according to our previous reports.[12,13]

Plasma enzyme activities and total bilirubin level

Total bilirubin concentration in plasma and the plasma alkaline phosphatase (ALP), alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) activities in samples were determined with commercially available kits (Zistshimi, Tehran, Iran).

Plasma nitrite and nitrate concentration

The measurement was performed according to Miranda *et al*.[19] Plasma samples were deproteinized by centrifugation through a 30-kDa molecular weight filter (Centricon Millipore, Bedford, USA) at 11 000 *g*. After loading the plates with samples (100 μL), addition of saturated solution of VCl3 (100 μL) to each well was rapidly followed by addition of the Griess reagent (100 μL). Sulfanilamide and naphthylethylenediamine dihydrochloride were applied for preparation of Griess reagent. The plates were incubated at 30°C for 30 min and then absorbance at 540 nm was measured using a standard plate reader. Fresh standard solutions of nitrate were included in each experiment.

Hepatic S-adenosylmethionine and S-adenosylhomocysteine

Liver specimens were snap frozen in the liquid nitrogen for the subsequent measurement of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH). At the time of measurement, liver tissues were homogenized in four volumes of 0.4 mol/L $HClO_4$, then centrifuged at 10 000 *g* for 20 min. The SAM and SAH levels in liver homogenates were determined by isocratic high-performance liquid chromatography with ultraviolet detection, as described previously.[20] The method provides rapid resolution of both compounds in a single run by direct injection of perchloric acid extracts so that sampling procedures and analytical errors can be reduced.

Total protein was measured using the Lowry protein assay.[21] All measurements are reported in nanomoles per milligram of protein to adjust for possible changes in milligrams of protein per gram of liver.

Type IV collagenase activity of liver

The MMP-2 activity was determined by gelatin zymography as previously described.[22] In brief, liver samples were homogenized in a glass homogenizer with lysis buffer (1% Triton X-100, 500 mmol/L, Tris/HCl, pH 7.6, 200 mmol/L NaCl, and 10 mmol/L $CaCl_2$) and centrifuged at 10 000 *g* for 15 min. The protein content was assayed by the Lowry assay. Twenty micrograms of protein was subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gel, impregnated with 1 mg/mL gelatin. After electrophoresis, gels were washed twice, for 30 min each time, in buffer, containing 1% Triton X-100, to displace SDS, and the gels were developed for 48 h in 50 mmol/L Tris buffer containing 5 mmol/L $CaCl_2$ (pH 7.4). The gels were stained with 1% Coomassie Brilliant Blue and then destained. Conditioned medium from 12-O-tetradecanoylphorbol-13-acetate (TPA)-stimulated HT1080 cells was used as a positive control. Using a UVI Pro gel documentation

system (GDS-8000 System; UVI photo MW, V.99 software, Cambridge, UK), quantitative evaluation of both surface and intensity of lysis bands, on the basis of gray levels, was performed and expressed as relative gelatinolytic activity.

Histological study

Formalin fixed, paraffin-embedded sections of the right and left liver lobes were cut at 3 μm and stained with hematoxylin–eosin (HE) and Masson's trichrome, then coded for blind reading by a single pathologist. Fibrosis was staged 0–4 based on Scheuer's scoring system.[23] The intensity of necroinflammatory activity and portal inflammation (0–4) were described based on modified hepatic activity index grading by Ishak *et al*.[24]

Statistical analysis

Results are expressed as mean ± SEM. Statistical evaluation of data was performed using the analysis of variances (ANOVA) followed by Tukey post hoc test. $P < 0.05$ was considered statistically significant.

Results

General characteristics

Chronic opioid receptor blockade did not change the survival rate, food intake or weight of cholestatic animals (Table 1).

The general characteristics of the animals in all four groups are shown in Table 1. Seven days after BDL or sham surgery, the survival rate in both groups of rats that underwent sham surgery was 100%. In fact, there were no significant differences between the saline- and naltrexone-treated control rats in any of the parameters, except that the naltrexone-treated control rats had a small decrease in the amount of their food intake. In contrast, 80% of the saline-treated BDL rats survived to 7 days. These rats had jaundice and the color of their urine was intense yellow compared to the weak-yellow urine of the sham groups. They did not have abdominal distension or ascites. Their food intake and bodyweight were significantly less than the sham groups.

Moreover, the 7-day survival of the naltrexone-treated BDL rats was the same as the saline-treated ones. There was no noticeable difference in the general appearance of these rats compared to the saline-treated BDL rats. They were jaundiced and had dark urine. Furthermore, chronic opioid receptor blockade did not influence the weight or the amount of daily food intake in BDL animals (Table 1).

Plasma bilirubin level and liver enzyme activity

Naltrexone-treated BDL animals had significant attenuation in plasma enzyme activity (Fig. 1).

After 1 week, plasma bilirubin and liver enzymes (ALT, ALP, GGT) were significantly elevated in BDL rats consistent with biliary obstruction compared with sham controls. Daily administration of naltrexone significantly attenuated the increase in plasma ALT, ALP and GGT activities. However, plasma bilirubin, a serum marker of cholestasis, was virtually identical in BDL–saline and BDL–naltrexone groups. Chronic opioid receptor blockade by naltrexone did not influence plasma enzyme activities and bilirubin level in the sham-operated groups (Fig. 1).

Plasma nitrite and nitrate concentrations

Chronic naltrexone administration prevented NO overproduction of cholestasis (Fig. 2).

Plasma nitrite and nitrate were measured as an indirect index of NO production. In the 7-day BDL rats, there was a significant increase in the combined nitrite and nitrate levels ($P < 0.001$). Chronic naltrexone administration had no effect on plasma nitrite and nitrate level in sham-operated rats, but significantly decreased it in the BDL group (64.9 ± 3.6 μmol/L *vs* 41.2 ± 3.0 μmol/L, BDL–saline and BDL–naltrexone, respectively, $P < 0.001$; Fig. 2).

Hepatic SAM and SAH levels

The SAM : SAH ratio of the liver improved by chronic opioid receptor blockade in cholestasis.

Liver SAH content increased after BDL. It was significantly different from sham-operated animals by the end of the first week.

Table 1 Subject characteristics (mean ± SEM)

	Sham–saline	Sham–naltrexone	BDL–saline	BDL–naltexone
Survival at day 7 (%)	100	100	80[a]	80[a]
Bodyweight (g)				
Day 0	245.6 ± 1.4	245.5 ± 1.8	246.6 ± 1.8	245.7 ± 1.2
Day 7	267.8 ± 1.7	269.0 ± 2.0	252.2 ± 1.8[a]	252.9 ± 1.4[a]
Food intake (g/day per rat)	21.08 ± 0.36	19.51 ± 0.16[b]	15.16 ± 0.38[a]	14.58 ± 0.59[a]
Liver/bodyweight (%)	3.24 ± 0.13	3.13 ± 0.13	4.86 ± 0.12[a]	4.96 ± 0.11[a]
Fibrosis	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.16	0.00 ± 0.00
Portal inflammation	0.00 ± 0.00	0.00 ± 0.00	1.87 ± 0.35[a]	1.37 ± 0.26[a]
Necroinflammatory score	0.00 ± 0.00	0.00 ± 0.00	4.87 ± 0.51[a]	3.86 ± 0.54[a]

BDL, bile duct ligation; sham, sham operation.

Groups consisted of saline-treated BDL rats (BDL–saline) or sham-operated rats (sham–saline); sham-operated rats treated chronically with naltrexone (sham–naltrexone); or BDL rats treated chronically with naltrexone at a dose of 20 mg/kg per day i.p for 7 consecutive days after surgery (BDL–naltrexone).

[a]$P < 0.01$ *vs* sham–saline; [b]$P < 0.05$ *vs* sham–saline.

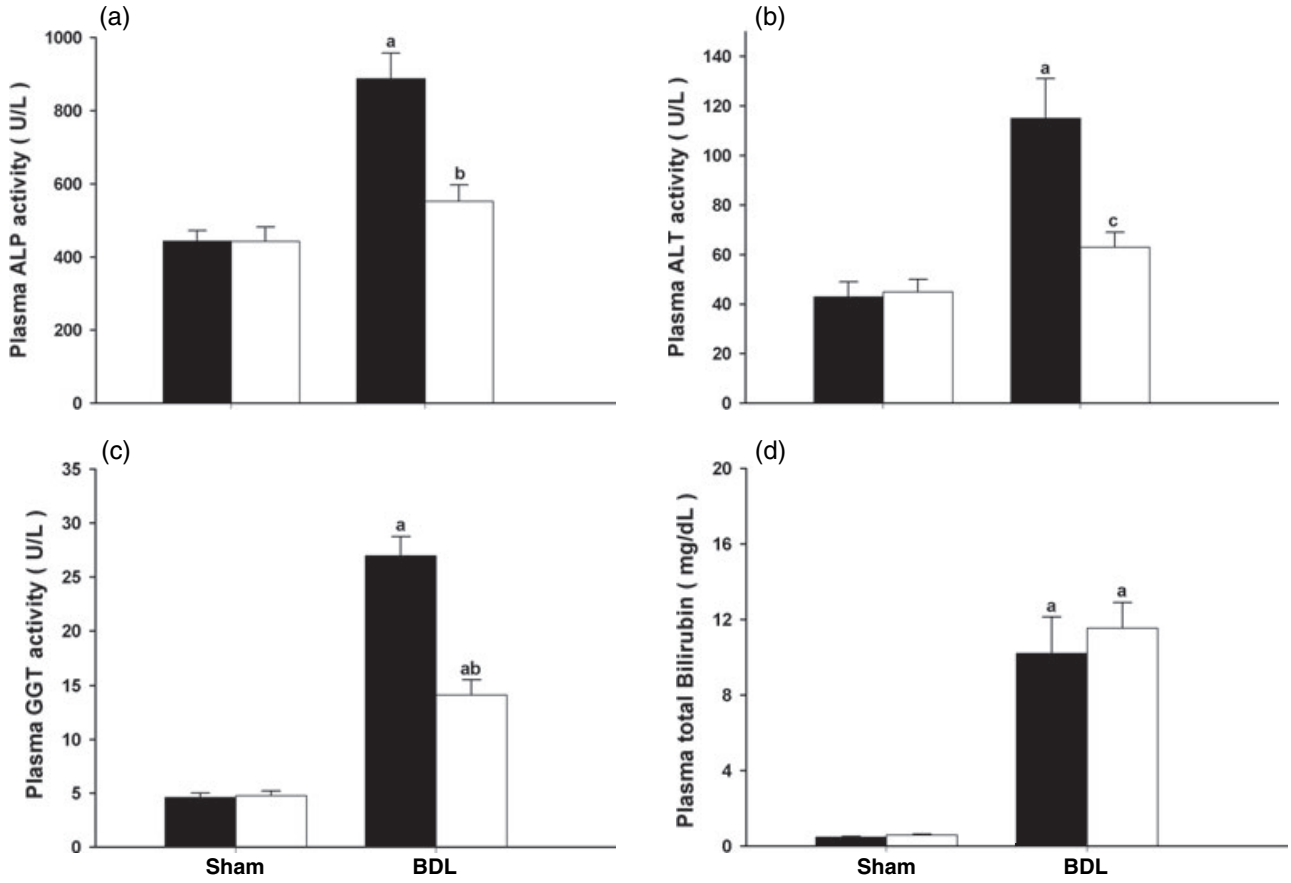


Figure 1 Plasma liver enzyme activity of (a) alkaline phosphatase (ALP); (b) alanine aminotransferase (ALT); (c) γ-glutamyl transpeptidase (GGT); and (d) bilirubin of plasma in bile duct-ligated (BDL) and sham-operated (sham) rats given (■) saline or (□) naltrexone. Data are given as mean + SEM ($n = 8$ for each group). [a]$P < 0.001$ *vs* sham–saline; [b]$P < 0.001$, [c]$P < 0.01$ *vs* BDL–Saline.

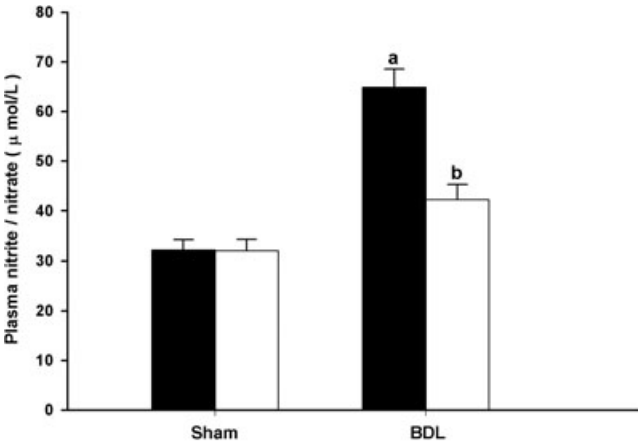


Figure 2 Plasma nitrite and nitrate levels in 7-day bile duct-ligated (BDL) and sham-operated (sham) rats given (■) saline or (□) naltrexone. Plasma nitrite and nitrate levels are increased at day 7 following BDL. Naltrexone treatment significantly inhibited this increase. Data are shown as mean + SEM ($n = 8$ for each group). [a]$P < 0.001$ *vs* sham–saline; [b]$P < 0.001$ *vs* BDL–saline.

Seven-day BDL also led to a modest reduction in liver SAM level (Table 2), but this did not reach statistical significance. The SAM : SAH ratio, expressed as hepatic methylation ratio, diminished by the seventh day of cholestasis and its level fell by 45% of the normal value (3.62 ± 0.46 *vs* 1.43 ± 0.11, sham–saline and BDL–Saline, respectively, $P < 0.001$;Fig. 3).

In contrast, the decrease in methylation ratio did not occur in 7-day BDL animals that were chronically treated with naltrexone throughout the study (Fig. 3). This was concomitant with a significant rise in liver SAM level (Table 2). Moreover, in BDL rats, liver SAH content also declined by naltrexone treatment to the level that was not significantly different from that of sham-operated groups (Table 2). There were no significant differences between the saline- and naltrexone-treated control rats in the mentioned parameters.

Type IV collagenase (MMP-2) activity

Chronic opioid receptor blockade decreased MMP-2 activity of the liver in BDL rats.

During zymography, MMP proenzyme is activated by denaturation and refolding, as a result, it can be visualized at the end of the assay (latent band). In this regard, total hepatic MMP-2 activity

Table 2 Hepatic concentration of SAM and SAH

	Sham–saline	Sham–naltrexone	BDL–saline	BDL–naltrexone
SAM (nmol/mg protein)	0.45 ± 0.04	0.52 ± 0.04	0.32 ± 0.03	0.51 ± 0.04[b]
SAH (nmol/mg protein)	0.13 ± 0.01	0.14 ± 0.01	0.22 ± 0.01[a]	0.17 ± 0.01

BDL, bile duct ligation; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; sham, sham operation.
Groups consisted of saline-treated BDL rats (BDL–saline) or sham-operated rats (sham–saline); sham-operated rats treated chronically with naltrexone (sham–naltrexone); or BDL rats treated chronically with naltrexone at a dose of 20 mg/kg per day i.p for 7 consecutive days after surgery (BDL–naltrexone).
[a]$P < 0.01$ *vs* sham–saline; [b]$P < 0.01$ *vs* BDL–saline.
. Values are expressed as .

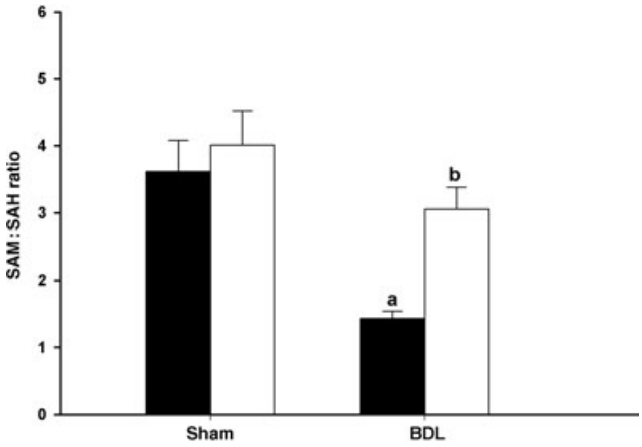
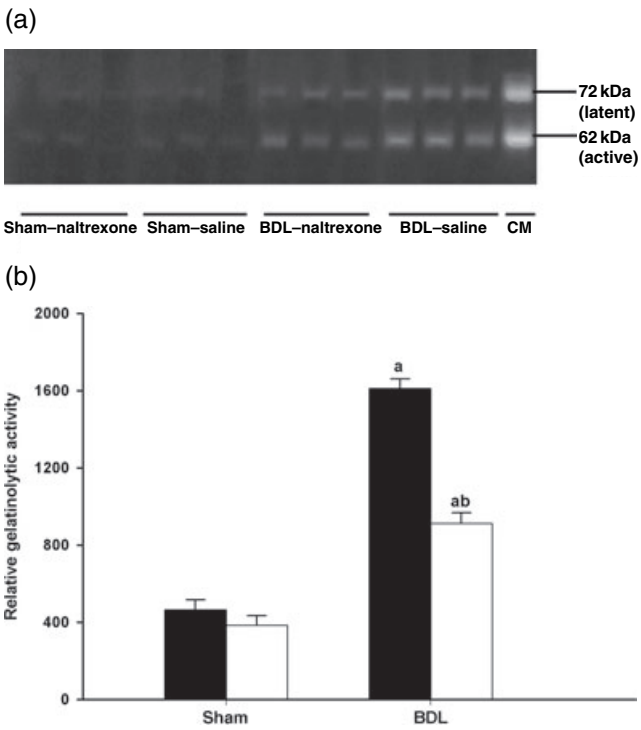


Figure 3 Liver S-adenosylmethionine (SAM) : S-adenosylhomocysteine (SAH) ratio in 7-day bile duct-ligated (BDL) and sham-operated (sham) rats given (■) saline or (□) naltrexone. Liver SAM : SAH ratio was decreased at the 7th day following BDL. This decrease was significantly inhibited by treatment with naltrexone (BDL–naltrexone). Data are given as means + SEM ($n = 8$ for each group). [a]$P < 0.01$ *vs* sham–saline; [b]$P < 0.05$ *vs* BDL–saline.



Figures 4 Matrix metalloproteinase-2 (MMP-2) activity of liver homogenates, evaluated after sodium dodecylsulfate–polyacrylamide gel electrophoresis and gelatin zymography. Quantitative analysis showed that the intensity of both the 72-kDa and 62-kDa bands increased significantly in the bile duct-ligated (BDL)–saline group, which was attenuated after opioid receptor blockade. Conditioned medium (CM) from HT1080 cells expressing active and latent forms of MMP-2 was included as a control for size comparison with sample gelatinolytic bands. Data are given as mean + SEM ($n = 8$ for each group). [a]$P < 0.001$ *vs* sham–saline; [b]$P < 0.01$ *vs* BDL–saline. Sham, sham-operated group.

(latent + active), which is assessed by gelatin zymography, is an appropriate estimate for the total MMP-2 protein expression.[25] As shown in Fig. 4, the degree of total MMP-2 activity increased more than threefold in cholestatic rats compared with controls ($P < 0.001$), and this was partially abrogated by naltrexone treatment in BDL rats (1610 ± 50 *vs* 912 ± 54, BDL–saline and BDL–naltrexone, respectively, $P < 0.001$). For both latent and active forms of MMP-2, statistically significant differences were observed between control animals *vs* 7-day saline-treated BDL animals ($P < 0.001$). Naltrexone treatment resulted in a significant decrease in the intensity of both active and latent bands (Table 3; $P < 0.01$).

Liver histology

Microscopic examinations of liver were not significantly different between naltrexone- and saline-treated groups in both BDL and sham-operated animals.

Morphologic evaluation of the liver in 7-day BDL rats revealed varying degrees of bile duct proliferation, inflammation and necrosis, almost without fibrosis. There is infiltration of inflammatory cells (polymorphonuclear cells and lymphocytes) in the cholestatic livers of both naltrexone- and saline-treated groups. Comparing the scores of portal inflammation and parenchymal necrosis, there was no significant difference between the naltrexone- and saline-treated BDL rats ($P > 0.05$; Table 1). In saline-treated BDL rats, two out of eight rats exhibited first-degree liver fibrosis (expansion of portal tract without linkage). However, no degree of fibrosis was detected in any of the naltrexone-treated BDL rats ($P > 0.05$). There was also no change in the liver histology of sham-operated rats by treatment (Table 1).

Table 3 Relative values of gelatinolytic activities in liver tissue protein extract (mean ± SEM)

	Sham–saline	Sham–naltrexone	BDL–saline	BDL–naltrexone
MMP-2 (active)	237 ± 24	225 ± 31	855 ± 32[a]	495 ± 29[ab]
MMP-2 (latent)	225 ± 30	157 ± 25	754 ± 35[a]	388 ± 44[ac]

BDL, bile duct ligation; MMP-2, matrix metalloproteinase-2.

Groups consisted of saline-treated BDL rats (BDL–saline) or sham-operated rats (sham–saline); sham-operated rats treated chronically with naltrexone (sham–naltrexone); or BDL rats treated chronically with naltrexone at a dose of 20 mg/kg per day i.p for 7 consecutive days after surgery (BDL–naltrexone).

[a]$P < 0.001$ *vs* sham groups; [b]$P < 0.01$, [c]$P < 0.05$ *vs* BDL–saline.

Discussion

The results presented here demonstrate that 7 days after BDL, liver MMP-2 activity increases, accompanied by an increase in plasma biochemical markers of liver injury. Chronic opioid receptor blockade significantly reduces the level of plasma liver enzyme and hepatic MMP-2 activities in BDL rats. These changes are parallel with a decrease in NO overproduction.We also determined, for the first time, a decrease in the SAM : SAH ratio in the liver, following cholestasis. Chronic naltrexone treatment could improve this ratio and increase hepatic SAM level in BDL rats.

The contribution of endogenous opioids to some of the manifestations of liver disease such as pruritus, ascitis and hyperdynamic circulation has been shown previously.[12,13,26,27] However, there are no data regarding the participation of endogenous opioids in the pathogenesis of liver injury in cholestasis. Data presented in the current study identified a new role for opioid system in the process of liver damage and its biochemical alterations, which occur in the cholestatic liver.

Plasma bilirubin level in obstructive cholestasis in the BDL model is modulated by several factors that seem to be an interplay of liver and kidney cells in order to prevent accumulation of toxic materials due to the biliary obstruction; thus, its level is a marker of cholestasis rather than liver injury. Alanine aminotransferase is a cytosolic enzyme of the hepatocytes, so its increased concentration in plasma reflects hepatocyte injury.[28] γ-Glutamyl transpeptidase and ALP are enzymes embedded in the hepatocyte plasma membranes; likewise, damage to the cell membranes and, thus, injury to the liver can raise their level in plasma.[28,29] Attenuation of liver enzyme activity by chronic opioid receptor blockade in the present study corroborates previous observations in which liver injury and increased hepatic enzyme activity have been reported following administration of exogenous opioid agonists.[14–16] The lack of significant changes in plasma bilirubin level in BDL rats by naltrexone treatment suggests that opioid blockade does not alter the degree of cholestasis while reducing liver enzyme activities that are associated with liver injury in this model.

In short-term BDL, the proteolytic degradation of extracellular matrix (ECM) by MMP is essential for a number of pathologic events, including the invasion of liver by mononuclear cells or, more importantly, the migration and activation of HSC.[11] The modulation of MMP by opioids has been demonstrated in some previous studies.[30,31] In a recent study on fibrosarcoma cell lines *in vitro*, we found that morphine could influence the production of MMP-2 via an NO-mediated mechanism (Shariftabrizi *et al.*, unpubl. data, 2005). In the current study, following chronic opioid receptor blockade in BDL rats we have observed attenuation in the total and active MMP-2 activities, which were concomitant with a decrease in NO metabolites. These findings also propose the involvement of an NO-mediated mechanism in modulation of hepatic MMP-2 by opioids.

However, the underlying mechanisms are ambiguous and some of the findings in the present study may contribute to this subject. In the present study, SAM : SAH ratios in livers of BDL rats were considerably lower in contrast to corresponding sham group, and increased by chronic naltrexone treatment. The importance of SAM production in the pathogenesis of liver injury has recently been well-characterized by Mato and his group.[32] S-adenosylmethionine is generated in the liver, using methionine and adenosine triphosphate. It is used in transmethylation reactions in which methyl groups are added to compounds and SAM is converted to SAH. S-adenosylhomocysteine is a competitive inhibitor of transmethylation reactions; hence, the SAM : SAH ratio regulates the majority of methylation reactions in the liver. S-adenosylmethionine, a ubiquitous methyl donor of hepatocytes, acts also as an intracellular signal that controls essential hepatic functions such as hepatocyte growth, differentiation and sensitivity to liver injury.[33] Thus, it has been suggested that a deficiency in hepatic SAM concentration contributes to the pathogenesis of liver cirrhosis.[32] In a landmark study, Mato and his group demonstrated that restoration of the SAM : SAH ratio attenuated CCl_4-induced liver injury and prevented DNA hypomethylation.[34,35] Moreover, it has been shown that chronic SAM treatment in BDL animals improves liver damage and decreases liver enzyme activities.[36] Accordingly, increased methylation ratio and liver SAM level by opioid blockade in BDL rats can reduce plasma enzyme activities and improve liver function.

In eukaryotic cells, the expression of genetic information is associated with the extent of DNA methylation.[37] This is similar to MMP-2-specific gene expression, which is also negatively influenced by its amount of methylation.[4,5] Because increased SAM : SAH ratio in liver enhances the degree of DNA methylation,[38] it can be postulated that naltrexone treatment can decrease total liver MMP-2 possibly by increasing the SAM : SAH ratio, increasing the amount of DNA methylation. However, further studies are recommended in this regard.

It has been shown that NO can inactivate hepatic methionine adenosyl transferase (MAT), the enzyme that produces SAM, by S-nitrosylation of the enzyme.[39] Thus, the improvement of liver SAM level and methylation ratio by naltrexone in BDL rats, may at least in part be due to the prevention of NO overproduction. Attenuation of NO production may also directly decrease MMP-2 activity[6,7] and modulate liver injury.

The observed improvements in the biochemical markers following naltrexone treatment were not concomitant with histological findings. This could be due to the level of sensitivity of the procedure. Microscopic examination is not sensitive enough to distinguish all changes that may take place in the liver. Furthermore, at the molecular level, a hepatic lesion starts long before it is histologically evident.[32] More importantly, these two events may not occur simultaneously. A histological effect might become evident at a later time point (i.e. up to day 28, when there is apparent liver fibrosis).

It has been proposed that both overproduction of opioids and retention due to impaired biliary excretion, may contribute to the accumulation of opioid peptides in liver diseases.[8,10] Liver is the main route for elimination of endogenous opioid peptides via their excretion into the bile.[8] Therefore, the accumulation of circulating opioids is clearly expected in different models of cholestasis in which there is an impaired hepatic excretion of bile. Consistent with this notion, the elevated plasma opioid peptides have been identified in animals and patients with cholestasis.[10,39,40] The increased circulating level of endogenous opioids along with present results on hepatoprotection by naltrexone treatment in BDL rats suggest the therapeutic potential of this agent for other models of cholestasis as well.

In summary, for the first time, the present study suggests that the endogenous opioid system contributes to liver injury in cholestasis. Our findings are of interest, and are potentially relevant clinically because naltrexone is used commonly to treat cholestasis-associated pruritus. The potential hapatoprotective effect of opioid receptor blockade has experimental and clinical implication in the attenuation of liver damage, which warrants further study.

Acknowledgments

We wish to express our thanks to Professor James D Finkelstein, Dr Matias Avila and Dr Ali Reza Mani for their support and pertinent comments. This work was supported by a grant from Forum for Dynamic Thoughts and Dean of Research, Tehran University of Medical Sciences (grant number: 131/8806).

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