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Effect of lovastatin on cholestatic liver injury induced by bile duct ligation in rats

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Abbreviations: CBD, common bile duct; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA

Abstract

The involvement of bile acids in hepatic damage under cholestasis and the effect of lowering bile acid level through the inhibition of microsomal 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by lovastatin was studied. Rats were divided into five groups: Sham operated control (group 1), common bile duct (CBD) ligation alone (group 2), choledococaval shunt operation (group 3), CBD ligation plus taurocholic acid injection (group 4), and CBD ligation plus lovastatin injection (group 5). Markers which reflect hepatic injury in serum in group 3 showed a greater increase than that in group 1. Serum total bile acid, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, 5'-nucleotidase and total bilirubin levels in group 4 showed greater increases than those in group 2. The levels of serum alanine aminotransferase, aspartate aminotransferase and cytosolic total bile acid in group 5 were lower than those in group 2. Serum total bile acid and microsomal HMG-CoA reductase levels in group 5 were not significantly lower than those in group 2. Rats in group 3 showed focal areas of hepatic necrosis associated with some infiltration of a few inflammatory cells. The abnormalities of the light microscopic structures of the liver of rats were more evident in group 4 than in group 2, and were preserved in group 5. According to the results, the choledococaval shunt operated rats resulted in hepatic damages. More hepatic damage was caused by administration of taurocholic acid under the cholestasis. Lovastatin, an inhibitor of HMG-CoA reductase, prevented the hepatic damage under the cholestasis. These experiments demonstrate that bile acid is one of the major factors which contribute to the damages observed in cholestasis, and that lowering cytosolic bile acid level through inhibition of HMG-CoA reductase is helpful in protecting the cholestatic damage.

Keywords: bile acid, cholestasis, 3-hydroxy-3-methylglutaryl CoA reductase, lovastatin

Introduction

In humans, cholestasis occurs in various diseases such as late stage of viral hepatitis, carcinoma of the bile duct, gallstones in the bile duct, primary biliary cirrhosis, sclerosing cholangitis, biliary atresia, alcoholic hepatitis, and during and after certain medications (Eddleston, 1994). In rats, cholestasis can be induced by common bile duct (CBD) ligation. CBD ligation in rats causes biochemical and morphological abnormalities in the liver such as inflammation, necrosis, fatty change, biliary hyperplasia, fibrosis, and cirrhosis (Kountouras *et al.*, 1984; Chang *et al.*, 1987; Kim *et al.*, 1989). The mechanism causing these abnormalities is not clear, although increased biliary hydrostatic pressure, retention of biliary constituents, and impairment of hepatocellular transport have been suggested (Hardison *et al.*, 1983). Biliary constituents contain bile acids which have several cytotoxic effects (Hardison *et al.*, 1983). The primary bile acids are synthesized in the liver from cholesterol (Mayes, 1993). In the biosynthesis of bile acids, the rate-limiting step is catalyzed by 7α-hydroxylase which prefers newly synthesized cholesterol as a substrate (Pandak *et al.*, 1990). Newly synthesized cholesterol can be reduced through inhibition of microsomal 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase by lovastatin (Mayers, 1993). Therefore, inhibition of HMG-CoA reductase by lovastatin may reduce the hepatic damage by reducing bile acid levels. No studies have been done on the effect of lowering bile acid level by the inhibition of HMG-CoA reductase using lovastatin on the hepatic damage induced by CBD ligation.

To evaluate the involvement of bile acids in hepatic damage under cholestasis and the effect of lovastatin on cholestatic damage the following experiments were conducted. In the serum and liver, the level of total bile acid and levels of various markers which are known to reflect hepatic injury were measured in cholestatic rats induced by CBD ligation in rats with choledococaval shunt operation which is characterized by complete biliary retention without high biliary hydrostatic pressure, in cholestatic rats induced by CBD ligation

plus taurocholic acid injection which is increased significantly during obstructive jaundice in humans, and in cholestatic rats induced by CBD ligation plus with lovastatin injection. Light microscopic studies of hepatic histopathology of these rats were also included herein.

Materials and Methods

Chemicals

β-NAD^+ (from yeast, grade III), triethanolamine HCl, DL-HMG-CoA, β-NADPH, sodium arsenite, tris(hydroxymethyl)aminomethane, 5,5'-dithio-bis(2-nitrobenzoic acid), taurocholic acid sodium salt, DL-dithiothreitol, diaphorase, 3α-hydroxysteroid dehydrogenase and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Bilirubin and cholesterol determination kits were purchased from Eiken Chemical Co. (Tokyo, Japan). Serum albumin determination kits was purchased from Yeong Dong Co. (Seoul, Korea). All other chemicals were of the highest purity available.

Animals

Normal male rats of the Sprague-Dawley strain, weighing between 320 and 350 g, were used in these experiments. Each experimental group consisted of 10 rats. Five rats in each group were used for the biochemical experiment, and the other 5 rats were used for the light microscopic examination. All animals were maintained on commercial pellets purchased from Sam Yang Food Co. (Wonju, Korea).

Treatment of the animal

In the sham operated control group (group 1), rats were sacrificed on the second day after the operation. In the CBD ligated group (group 2), rats were sacrificed on the second day after the ligation. In choledococaval shunt operation group (group 3), CBD and right jugular vein were connected with medical grade silicon tubing. On the second day after the operation, the rats were sacrificed. In CBD ligation plus taurocholic acid treated group (group 4), according to the method of Ogawa *et al.* (1990), a dose of 450 μmol of taurocholic acid per kg of body weight was injected through intravenously at the time of CBD ligation. In CBD ligation plus lovastatin treated group (group 5), according to the modified method of Low *et al.* (1992), a dose of 18.75 mg of lovastatin per 100 g of body weight was injected twice intraperitoneally, first at the time of CBD ligation and second at 24 h after the first injection. Then, the rats were sacrificed at 24 h after second injection of lovastatin.

The rats were anesthetized lightly with ether for surgery or being sacrificed and they were fasted for 12 h prior to sacrifice.

For CBD ligation, the CBD was exposed through a middle line incision. After double ligation of CBD, the mid point was cut. The sham operation was performed in the same manner without CBD ligation.

Cell fractionation

The rats were anesthetized lightly with ether, blood was collected from abdominal aorta and liver was perfused through the portal vein with 0.25 M sucrose, and the serum and liver were obtained. The obtained liver was rinsed in 0.25 M sucrose, and the surface was wiped dry. Cytosol and microsome was obtained by sucrose density gradient centrifugation (Kwak and Kwak,1986).

Histology

After buffered formalin fixation, the liver slices were embedded in a standard manner, and stained with haematoxylin and eosin. The microscopic examination was interpreted by one experienced pathologist.

Assays

Total bile acid level was measured using a spectrophotometer (DU 650, Beckman) according to the method of Mashige *et al.* (1981). 5'-Nucleotidase activity was assayed, by the method of Campbell (1962), and HMG-CoA reductase activity was measured by the method of Shapiro and Rodwell (1971). Alanine aminotransferase and aspartate aminotransferase activities were determined according to the method of Reitman-Frankel (1957). Alkaline phosphatase activity was determined according to the method of Kind and King (1954), and γ-glutamyl transpeptidase activity was by the method of Orlowski and Meister (1963).

Determination of protein

The serum total protein and protein concentrations of each subcellular fractions were determined by the biuret method (Gornall *et al.*,1949) using bovine serum albumin as the calibrator.

Statistical analysis

Values were expressed as mean ± S.D. Statistical evaluation of significant difference between means was performed by the Student's *t*-test. *P* values of ≤ 0.05 were considered significant.

Results and Discussion

The involvement of bile acids in hepatic damage under cholestasis and the effect of lowering bile acid level through the inhibition of microsomal HMG-CoA reductase by lovastatin were studied.

As shown in Table 1 and 2, markers of hepatic injury such as serum alanine and aspartate aminotrans-

Table 1. Effects of lovastatin administration on serum markers of hepatic injury in cholestatic rats.

	Sham operation (Group 1)	CBD ligation (Group 2)	Choledococaval shunt (Group 3)	CBD ligation + taurocholic acid (Group 4)	CBD ligation + lovastatin (Group 5)
Alanine aminotransferase (Karmen unit ml^{-1})	32 ± 10	470 ± 169[c]	400 ± 45[c]	812 ±282[c,d]	239 ± 46[c,d]
Aspartate aminotransferase (Karmen unit ml^{-1})	111 ± 28	1,100 ± 285[c]	1,336 ± 158[c]	1,406 ±240[c]	650 ± 240[b,d]
Alkaline phosphatase (μmol phenol min^{-1} ml^{-1})	2.61 ± 0.87	10.52 ± 2.03[c]	7.40 ± 0.43[c,d]	18.40 ± 6.99[b,d]	8.37 ± 1.25[c]
γ-Glutamyl transpeptidase (μmol p-nitroaniline min^{-1} ml^{-1})	3.16 ± 0.53	61.40 ± 24.09[c]	43.41 ± 13.36[c]	196.11 ± 62.68[c,e]	52.26 ± 17.06[c]
5'-Nucleotidase (nmol Pi min^{-1} ml^{-1})	0.37 ± 0.18	3.20 ± 0.66[c]	1.9 3 ± 0.15[c,b]	5.05 ± 1.05[c,b]	2.78 ± 0.55[c]
Total bilirubin (mg dl^{-1})	0.28 ± 0.05	9.93 ± 3.09[c]	9.50 ± 2.98[c]	15.00 ± 4.36[c,d]	9.97 ± 2.16[c]
Total protein (g dl^{-1})	7.61 ± 0.40	7.88 ± 0.53	7.32 ± 0.27	7.53 ± 0.61	7.41 ± 0.56
Albumin (g dl^{-1})	3.74 ± 0.29	3.43 ± 0.43	3.33 ± 0.21	3.52 ± 0.37	3.47 ± 0.25
Total cholesterol (mg dl^{-1})	54 ± 9	266 ± 55[c]	251 ± 43[c]	350 ± 109[c]	272 ± 72[c]
Total bile acid (μmol l^{-1})	4.8 ± 1.6	428.4 ± 20.5[c]	400.9 ± 69.9[c]	466.7 ± 18.2[c,d]	387.7 ± 50.3[c]

Values are means ± SD with 5 rats in each group. Animal groups were described in the text.
Values were significantly different from that of group 1 ([b] $P<0.01$; [c] $P<0.001$) and from that of group 2 ([d] $P<0.05$; [e] $P<0.01$).

Table 2. Effects of lovastatin administration on tissue markers of hepatic injury and hepatic microsomal HMG-CoA reductase activity in cholestatic rats.

	Sham operation (Group 1)	CBD ligation (Group 2)	Choledococaval + shunt (Group 3)	CBD ligation + taurocholic acid (Group 4)	CBD ligation + lovastatin (Group 5)
Cytosolic alanine aminotransferase (Karmen unit mg $protein^{-1}$)	644 ± 61	553 ± 95	611 ± 59	535 ± 109	599 ±78
Cytosolic aspartate aminotransferase (Karmen unit mg $protein^{-1}$)	680 ± 127	579 ± 49	661 ± 83	593 ± 57	614 ±55
Cytosolic alkaline phosphatase (μmol phenol min^{-1} mg $protein^{-1}$)	2.44 ± 0.70	8.10 ± 3.07[b]	4.65 ± 0.56[c,d]	10.03 ± 4.61[b]	9.57 ± 2.98[c]
Cytosolic γ-glutamyl transpeptidase (μmol p-nitroaniline min^{-1} mg $protein^{-1}$)	0.28 ± 0.15	0.61 ± 0.25[a]	0.29 ± 0.03[d]	0.46 ± 0.23	0.48 ± 0.21
Cytosolic 5'-Nucleotidase (nmol Pi min^{-1} mg $protein^{-1}$)	3.58 ± 0.36	6.92 ± 1.15[c]	6.25 ± 1.45[b]	10.86 ± 2.83[c,d]	5.23 ± 1.24[a]
Cytosolic total bile acid (μmol 100 g wet $live^{-1}$)	16.31 ± 7.93	29.41 ± 4.22[a]	24.56 ± 7.73	28.54 ± 5.46[a]	14.99 ± 4.81[e]
Total cholesterol (mg g wet $liver^{-1}$)	4.48 ± 0.26	4.90 ± 0.75	4.52 ± 0.35	4.41 ± 1.00	4.06 ± 0.72
Microsomal HMG-CoA reductase (nmol CoA formed min^{-1} mg $protein^{-1}$)	0.81 ± 0.09	0.94 ± 0.11	0.88 ± 0.11	0.87 ± 0.11	0.80 ± 0.09

Values are means ± SD with 5 rats in each group. Animal groups were described in the text.
Values were significantly different from that of group 1 ([a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$) and from that of group 2 ([d] $P<0.05$; [e] $P<0.01$).

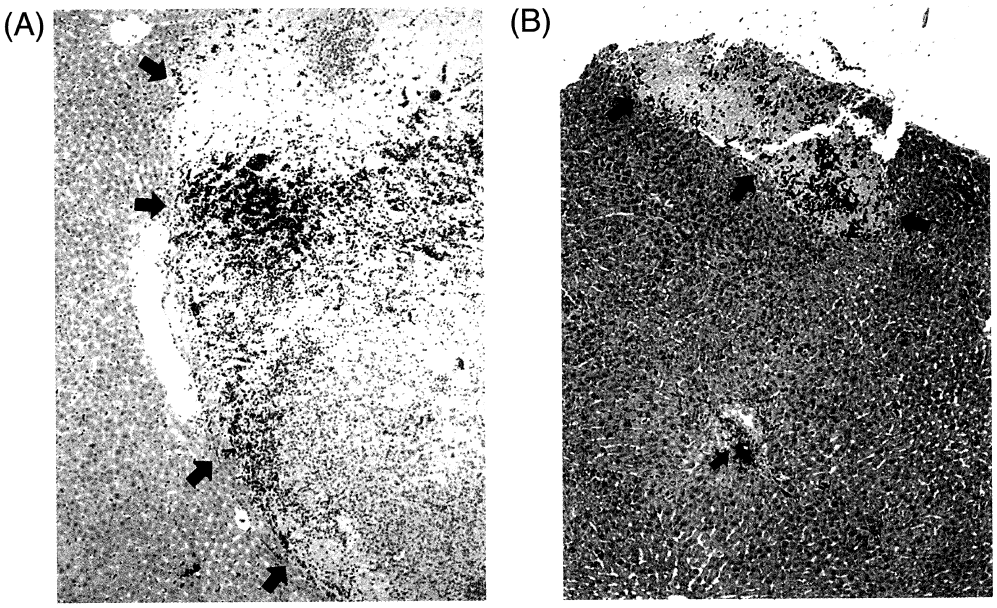

Figure 1. (A) Histological features of liver from rat with CBD ligated group. The liver shows a massive lobular necrosis with marked inflammatory cell infiltrate and hemorrhage in the necrotic area. (B) Liver from rat with choledococaval shunted group. There are area of flank necrosis with hemorrhage, mild pericholangitis and piecemeal necrosis in parenchyma. The prominence of Kupfer's cells is also visible. Closed arrows indicate necrosis with hemorrhage and inflammation (haematoxylin and eosin stain, original magnification x 40).

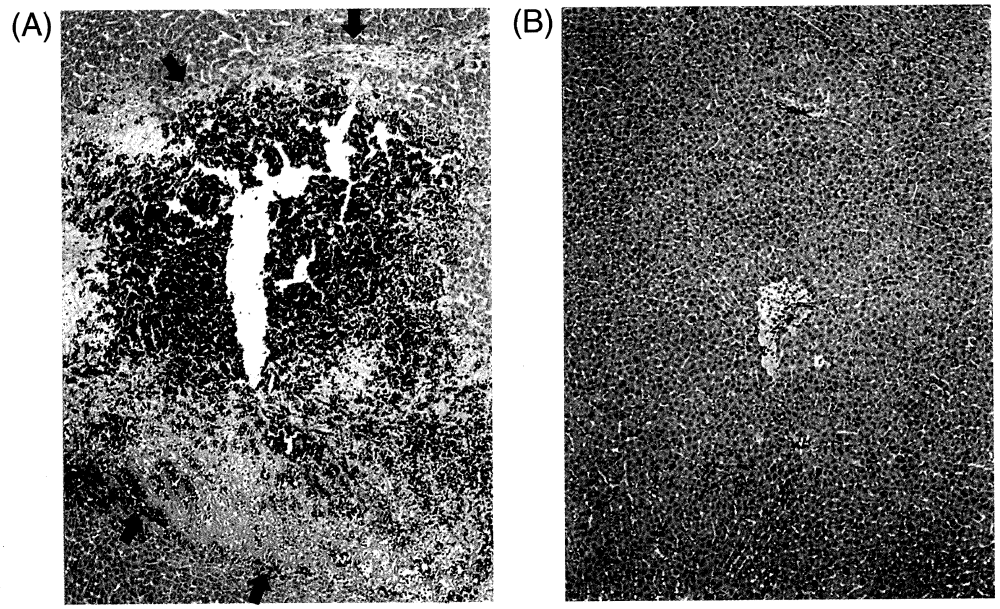

Figure 2. (A) Histological features of liver from cholestatic rat with taurocholic acid administered group. The liver shows a massive lobular necrosis with severe hemorrhage and collection of inflammatory cells in the border of necrotic and viable areas. (B) Liver from cholestatic rat with lovastatin administered group. There are small areas of mild collection of inflammatory cell in the parenchyma. Closed arrows indicate necrosis with hemorrhage and inflammation. Open arrows indicate piecemeal necrosis (haematoxylin and eosin stain, original magnification x 40).

ferases, total bilirubin, total cholesterol and total bile acid levels, and serum, and hepatic cytosolic alkaline phosphatase and 5'-nucleotidase activities were increased significantly from the rats of both the CBD ligation (group 2) and in the choledochocaval shunt (group 3) groups. However, the extent of various change which reflect hepatic injury such as serum and hepatic cytosolic alkaline phosphatases, and serum 5'-nucleotidase were smaller in group 3 than in group 2. The microscopic examination showed less severe changes in group 3 than in group 2 including the size of necrotic areas and the degree of infiltration of inflammatory cells in the area of necrosis (Figure 1A, B).

The result obtained by CBD ligation and choledochocaval shunt operation are similar each other in that bile concentration in the liver increases with retention of bile, however, they are dissimilar in the following aspects. In the CBD ligation, biliary hydrostatic pressure as well as hepatocellular tight junction permeability increases markedly by 6 h (Hardison *et al.*, 1983; Toyota *et al.*, 1984; Ogawa *et al.*, 1990). These changes promote regurgitant flux of biliary constituents across either the hepatocyte

basolateral membrane or through tight junctions back into the blood. In the choledochocaval shunt operation, retained biliary constituents exit, albeit at a faster rate, via a normal biliary pathway (Hardison *et al.*, 1983). Direction of bile acid transport in the choledochocaval shunt operation is likely to be normal since bile flow is unobstructed, and biliary hydrostatic pressure and tight junctional permeability remain low (Toyota *et al.*, 1984). Therefore, the above results of elevated alkaline phosphatase and 5'-nucleotidase activities indicate that factors other than bile acids contribute to the abnormalities observed in cholestasis induced by CBD ligation.

Markers which reflect hepatic injury such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, 5'-nucleotidase activities and total bilirubin levels in serum in group 3 showed greater increases than those in group 1 (sham operated control group) (Table 1). Serum total cholesterol and total bile acid, and hepatic cytosolic total bile acid levels also increased more in group 3 than in group 1 (Tables 1 and 2). Under the light microscopic examination, rats in group 3 showed focal areas of hepatic necrosis associated with infiltration of a few inflammatory cells (Figure 2B). Choledococaval shunt operated in rats, the group 3 which are characterized by complete biliary retention without high biliary hydrostatic pressure, had hepatic damages. These results indicated that simple retention of biliary constituents including bile acid contributed to the abnormalities observed in cholestasis which was induced by CBD ligation.

Bile salt can damge cells in a variety of tissues (Mekhjian and Phillips, 1970; Coleman and Holdworth, 1975; Safaie Shirazi *et al.*, 1975; Duane and Wiegand, 1980). Infusion of hydrophobic bile salts causes cholestasis in isolated perfused rat liver (Drew and Priestly, 1978; Kitani *et al.*, 1986), and ingestion or infusion of the bile salts produced hepatocellular necrosis both in experimental animals and in humans (Miyai *et al.*, 1971; King and Schoenfield, 1972; Palmer, 1972; Fisher *et al.*, 1982; Okun *et al.*, 1982). Toxicity of hydrophobic bile salts may be responsible in part for the hepatic necrosis which is accompanied by obstructive cholestasis (Greim *et al.*, 1972; Palmer, 1972).

Serum total bile acid, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, 5'-nucleotidase and total bilirubin levels of rats in group 4 (CBD ligation plus taurocholic acid treated group) showed greater increases than those in group 2 (Table 1). The abnormalities of the microscopic structures of the liver of rats in group 4 were aggravated more than in group 2 (Figures 1A and 2A). In rats under cholestasis, hepatic damage was aggravated by administration of taurocholic acid which increased significantly during obstructive jaundice in humans (Nishimura *et al.*, 1985). These results demonstrate that bile acid is one of the major factors contributing to the damages observed in cholestasis. The levels of serum alanine aminotransferase, serum aspartate aminotransferase and hepatic cytosolic total bile acid in group 5 were lower than those in group 2 (Tables 1 and 2). Serum total bile acid and microsomal HMG-CoA reductase levels in group 5 were not significantly lower than those in group 2 (Tables 1 and 2). The abnormalities of the hepatic microscopic structures of rats were less severe in group 5 (CBD ligation plus lovastatin treated group) than in group 2 (Figures 1A and 2B). Viewed from the above results, lovastatin, an inhibitor of HMG-CoA reductase, prevented the hepatic damage under the cholestasis. Thus, these experiments demonstrate that lowering hepatic cytosolic bile acid level through the inhibition of HMG-CoA reductase is helpful in protecting the cholestatic damage.

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