Effect of Rosuvastatin on Cholestasis-Induced Hepatic Injury in Rat Livers

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ABSTRACT: Recent studies reported that 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors have pleotropic effects independent of their lipid-lowering properties. The present study was undertaken to determine whether treatment with rosuvastatin (RO) would be beneficial in a rat model of bile duct ligation (BDL). Animals were divided into three groups: a sham group (group I), a BDL group treated with vehicle (group II), and a BDL group treated with RO (10 mg/kg) (group III). Serum levels of total bilirubin, γ-glutamyl transpeptidase, alanine aminotransferase, and aspartate aminotransferase decreased significantly in group III when compared to group II. Lipid peroxides and NO levels of group III were found to be significantly lower than those of group II. Antioxidant enzymes (superoxide dismutase, glutathione-*S*-transferase, and catalase) activity in liver tissues markedly decreased in group II, whereas treatment with RO preserved antioxidant enzyme activity. DT-diaphorase activity in group II was significantly higher than that in group III. The histopathological results showed multiple numbers of newly formed bile ductules with inflammatory cells infiltration in group II. These pathological changes were improved in group III. Our data indicate that RO ameliorates hepatic injury, inflammation, lipid peroxidation and increases antioxidant enzymes activity in rats subjected to BDL. RO may have a beneficial effect on treatment of cholestatic liver diseases. © 2010 Wiley Periodicals, Inc. J Biochem Mol Toxicol 24:89–94, 2010; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10:1002/jbt.20315

KEYWORDS: **Biliary obstruction; Bile duct ligation; Cholestasis; Inflammation; Lipid peroxidation; Oxidative stress; Rosuvastatin**

INTRODUCTION

Cholestasis is a condition caused by either a functional defect in bile formation at the level of the hepatocyte or from impairment in bile secretion and flow at the bile duct level. At the hepatocyte level, hormones and drugs can cause noninflammatory cholestasis. Proinflammatory cytokines (induced by infection, alcohol, or drugs) cause inflammatory cholestasis. At the bile duct level, progressive bile duct destruction leading to primary biliary cirrhosis and bile duct obstruction (by stenoses, stones, or tumors) interrupts bile flow. This leads to intracellular accumulation of bilirubin, bile acids, and cholesterol, causing stimulation of proinflammatory cytokines production and apoptosis enhancement that leads to hepatocellular damage [1,2]. In rats, common bile duct ligation produces a well-established experimental model of acute obstructive jaundice, progression of biliary fibrosis to cirrhosis, and secondary biliary cirrhosis. Bile duct ligation (BDL) produces a combined model of cholemia and parenchymal liver disease [3].

Statins, effective cholesterol-lowering agents, inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, a key enzyme that catalyzes the rate-limiting step within the cholesterol biosynthetic pathway. Several studies have shown that statins may also exert effects beyond their lipid-lowering properties. These pleotropic (nonlipid) actions include antioxidant effects [4], anti-inflammatory effects [5], upregulation of endothelial nitric oxide synthase [6], osteogenic effect [7], inhibition of platelet adhesion and aggregation [8], and normalization of sympathetic outflow [9]. Moreover, statins have shown promising results when used in combination with ursodeoxycholic acid in treatment of primary biliary cirrhosis [10].

Rosuvastatin (RO), a relatively new HMG-CoA reductase inhibitor, has exhibited a more potent affinity for the active site of HMG-CoA reductase than other

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statins. In addition, the cytoprotective action of rosuvastatin against ischemic injury has been clearly documented [11–14]. In rats, the hepatic uptake of rosuvastatin was found to be more selective and efficient than that seen with pravastatin or simvastatin [15]. It is thought likely that rosuvastatin is also distributed principally to the liver in humans, as shown by its high proportion of nonrenal clearance (>70%) [16]. Metabolism plays only a minor role in the elimination of the drug [17]. The cytochrome P450 (CYP) isoenzyme CYP3A4 has no significant role in the metabolism of rosuvastatin, with CYP2C9 thought to be the isoenzyme principally responsible for the minimal metabolism that occurs via the CYP system [17,18]. Hence, we found that rosuvastatin is an interesting target to be investigated for its protective effects in biliary obstruction induced-injury and inflammation in the liver.

MATERIALS AND METHODS

Chemicals and Drugs

Rosuvastatin was supplied by Astrazeneca (Cairo, Egypt). All the chemicals were analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals and Treatments

Twenty-four adult male Sprague–Dawley rats, weighing 200–250 g, were housed at cages in a temperature-controlled ($25 \pm 1^{\circ}$C) environment and provided free access to pelleted food and purified drinking water ad libitum. The animal experiments described shortly comply with the ethical principles and guidelines for the care and use of laboratory animals adopted by the National Egyptian Community. The animal experimental protocols were approved by the Al-Azhar University Committee.

The rats were divided into three groups (eight in each group) as follows:

Group I: Sham-operated group
Group II: Bile duct ligated group receiving vehicle (BDL + vehicle).
Group III: BDL group receiving rosuvastatin (BDL + RO).

Rosuvastatin was suspended in 10% Tween 20 solution and administered at a dose of 10 mg/kg of body weight orally for 7 days, starting from the third day after bile duct ligation.

Ten days after surgery, blood was collected from animals by retro-orbital puncture. Animals were then killed by cervical dislocation. The liver was rapidly removed for biochemical and histological examination.

Induction of Biliary Obstruction

Rats were anesthetized with sodium pentobarbital 35 mg/kg i.p., and the common bile duct was exposed and ligated by double ligatures with suture silk. The first ligature was made below the junction of the hepatic ducts, and second ligature was made above the entrance of the pancreatic ducts. Finally, the common bile duct was resected between the double ligatures. In sham-operated rats, an incision was made in the abdomen, which was then closed without any treatment.

Liver Function Tests

Plasma concentrations of bilirubin aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transpeptidase (γ-GT) were measured as indicators of hepatic injury using standard diagnostic kits (Quimica Clinica Aplicada S.A., Amposta, Spain).

Determination of Lipid Peroxides Level

Thiobarbituric acid reactive substances were measured spectrophotometrically using thiobarbituric acid [19].

Evaluation of Antioxidant Enzymes Activity

The liver was weighed and homogenized for evaluation of the following antioxidant enzymes activity.

Determination of Glutathione-S-Transferase Activity

Glutathione-S-transferase (GST) activity toward 1-chloro-2,4-dinitrobenzene in the presence of glutathione as cosubstrate was examined spectrophotometrically at 25°C. The enzyme activity was determined by monitoring change in absorbance at 340 nm [20].

Determination of Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by calculating the difference between autooxidation of pyrogallol alone and in the presence of homogenate that contained SOD [21].

Determination of DT-Diaphorase (Quinone Reductase) Activity

DT-diaphorase activity was assayed spectrophotometrically in the presence of 2,6-dichloroindophenol and nicotinamide adenine dinucleotide reduced form. Absorbance was measured at 600 nm [22].

TABLE 1. Effect of Rosuvastatin on the Serum Level of Bilirubin, γ-GT, ALT, and AST in Rats

Groups	*Bilirubin (mg/dL)*	*γ-GT (U/L)*	*ALT (IU/mL)*	*AST (IU/mL)*
Sham	11.45 ± 0.92	3.603 ± 0.57	38.25 ± 1.19	104.63 ± 9.22
BDL + vehicle	135.92 ± 12.03^a	10.81 ± 0.45^a	79.51 ± 15.53^a	178.95 ± 15.26^a
BDL + RO	28.46 ± 1.48^b	7.57 ± 0.82^b	36.63 ± 1.23^b	121.14 ± 5.56^b

Results are expressed as the mean ± SEM, $n = 8$ per group.
Sham: sham-operated rats, BDL: bile duct ligated rats, RO: rosuvastatin.
[a] Significantly different from the sham group at $P < 0.05$.
[b] Significantly different from the BDL + vehicle group at $P < 0.05$.

Determination of Catalase Activity

Catalase (CAT) activity was determined based on the decrease in light absorption at 240 nm because of the decomposition of hydrogen peroxide by catalase [23].

Determination of Nitric Oxide

Nitrite is a stable, nonvolatile breakdown product of nitric oxide (NO) that can be measured *in vitro*. Nitrite level was determined spectrophotometrically in cell-free supernatants from liver homogenate based on the Griess reaction [24]. Absorbance was read at 543 nm. The nitrite concentration was calculated from a standard curve and expressed as micromolar.

Determination of Total Protein

The protein content was measured according to the method of Lowry et al. using bovine serum albumin as standard [25].

Histological Examination

Autopsy samples were taken from the liver of rats in different groups then fixed in 10% formol saline for 12 h. Serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used. Specimens were cleared in xylene embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by a sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for histopathological examination through the light microscope [26].

Statistical Analysis

Results are expressed as the mean ± SEM, and different groups were compared using one way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons.

RESULTS

Effect of Rosuvastatin on BDL-Induced Alteration in Liver Function Tests

In the sham group, the mean levels of bilirubin, γ-GT, ALT, and AST were 11.45 ± 0.92 mg/dL, 3.603 ± 0.57 U/L, 38.25 ± 1.19, 104.63 ± 9.22 IU/mL, respectively. At the end of the experiment, bilirubin and the enzymes levels in the BDL + vehicle group were significantly increased. RO decreased bilirubin and enzymes level by 79.06%, 29.97%, 53.93%, and 32.30% respectively (Table 1).

Antioxidant Properties of Rosuvastatin

Compared with the sham group, the activity of antioxidant enzymes (SOD and CAT) in liver tissues decreased significantly when rats were subjected to BDL. GST activity decreased markedly but with no significant difference. Treatment with RO preserved antioxidant enzymes activity. On the other hand, quinone reductase activity increased in the BDL + vehicle group when compared with the sham group and decreased in the BDL + RO group significantly (Table 2).

TABLE 2. Effect of Rosuvastatin on Superoxide Dismutase (SOD), Glutathione-S-Transferase (GST), Catalase (CAT), and DT-Diaphorase Activities in Rats

Groups	*SOD (U/mg Protein)*	*GST (nmol/min/mg Protein)*	*CAT (U/mg Protein)*	*DT-Diaphorase (nmol/min/mg Protein)*
Sham	64.54 ± 4.3	163.79 ± 5.41	2.239 ± 0.47	26.38 ± 3.63
BDL + vehicle	42.25 ± 2.1^a	139.92 ± 2.57	0.795 ± 0.07^a	48.89 ± 4.73^a
BDL + RO	66.64 ± 4.65^b	167.73 ± 11.34^b	2.385 ± 0.1^b	25.49 ± 3.08^b

Results are expressed as the mean ± SEM, $n = 8$ per group.
Sham: sham-operated rats, BDL: bile duct ligated rats, RO: rosuvastatin.
[a] Significantly different from the sham group at $P < 0.05$.
[b] Significantly different from the BDL + vehicle group at $P < 0.05$.

TABLE 3. Effect of Rosuvastatin on Lipid Peroxides and Nitric Oxide in Rats Liver Homogenate

Groups	*Lipid Peroxides (nmol/g Tissue)*	*NO (μM)*
Sham	395.6 ± 8	10.78 ± 1.06
BDL + vehicle	505.3 ± 30.3^a	44.09 ± 7.06^a
BDL + RO	403 ± 21.6^b	18.99 ± 6.79^b

Results are expressed as the mean ± SEM, $n = 8$ per group.
Sham: sham-operated rats, BDL: bile duct ligated rats, RO: rosuvastatin.
[a]Significantly different from the sham group at $P < 0.05$.
[b]Significantly different from the BDL + vehicle group at $P < 0.05$.

Effect of Rosuvastatin on Lipid Peroxidation and Inflammation

Lipid peroxides level was found to be significantly lower in the BDL + RO group than the BDL + vehicle group. Liver level of NO increased in the BDL + vehicle group and decreased significantly in the BDL + RO group. No significant difference was found between the BDL + RO group and the sham group concerning these two parameters (Table 3).

Histopathological Findings

Histological liver changes were analyzed at the end of the experiment. There was no histopathological alteration, and the normal histological structure of the central vein and surrounding hepatocytes were recorded in the sham group (Figure 1A).

Concerning liver of the BDL + vehicle group, hyperplasia of the bile ducts with multiple numbers of newly formed bile ductules was observed in the portal area and extended to the adjacent hepatic parenchyma in between the hepatocytes in association with severe congestion of the portal vein. Mononuclear leucocytes inflammatory cells infiltration was noticed in between the newly formed bile ductules (Figure 1B). Severe dilatation and congestion were recorded in the central vein associated with focal necrosis as well as focal leucocytes inflammatory cells infiltration in the adjacent hepatocytes (Figure 1C). The liver of the BDL + RO group showed improvement in the histological findings appearing as a decrease in congestion of portal vein, lower number of newly formed bile ductules, and fewer inflammatory cells infiltration (Figure 1D).

DISCUSSION

Biliary obstruction is associated with an intense state of oxidative stress. Cholestasis per se reduces antioxidative capacities in liver mitochondria in bile duct ligated rats [27]. Accumulation of hydrophobic bile acids and inflammatory cells in the liver tissue may cause increased production of free radicals in biliary obstruction [28]. Bile acids especially enhance reactive oxygen species released by polymorphonuclear leukocytes [29]. In addition, intraluminal bile salt deficiency in extrahepatic biliary obstruction results in vitamin E

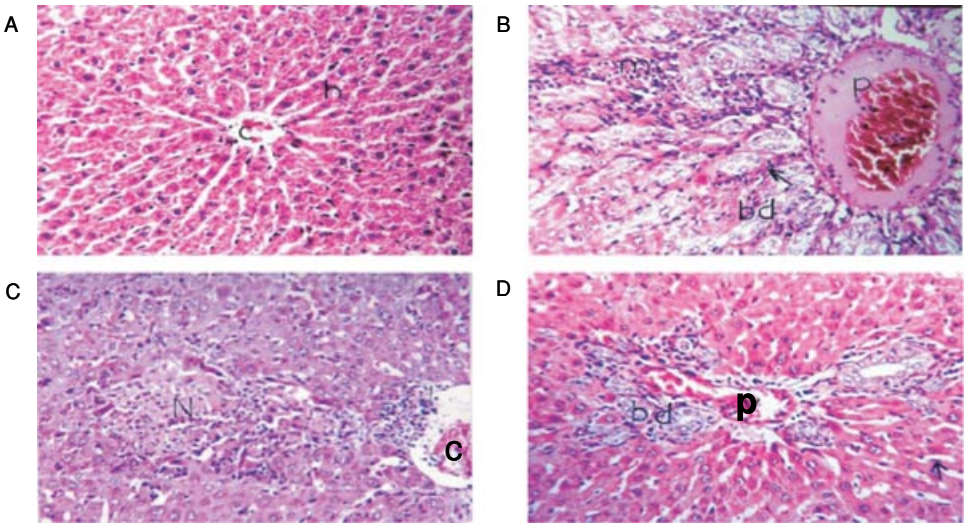

FIGURE 1. Hematoxylin- and eosin-stained sections (×64) showing effect of rosuvastatin (RO) on histological liver changes induced by bile duct ligation (BDL). (A) Sham: normal histological structure of central vein (c) and hepatocytes (h). (B) BDL + vehicle: congested portal vein (p) with inflammatory cells infiltration (m and arrow) in between multiple number of newly formed bile ductules (bd). (C) BDL + vehicle: focal necrosis (N) and focal inflammatory cells infiltration adjacent to the congested central vein (c). (D) BDL + RO: improvement of the histological findings showed as decrease congestion of portal vein (p), lower number of newly formed bile ductules (bd), fewer inflammatory cells infiltration (arrow).

malabsorption [30]. Accordingly, hepatic tissue levels of lipid peroxides were increased in bile duct ligated rats [31].

In this study, we investigated the effect of the most recent HMG-CoA reductase inhibitor, rosuvastatin, on biliary obstruction-induced oxidative stress associated with decreased activity of different enzymes with antioxidant properties. The results indicate that RO improved oxidative stress. Moreover, RO did not only decrease the parameters of liver injury induced by bile duct ligation but it was able to protect against lipid peroxidation. Ajith et al. suggested that the protective mechanism of RO in vitro can be correlated with the reducing equivalent donating property or direct hydroxyl radical scavenging activity of the drug but not superoxide anion-scavenging activity [32]. It was demonstrated that RO upregulates glutathione-synthesizing enzymes [33].

During bile duct obstruction and subsequent cholestasis, increased concentrations of bile acids and toxins in the liver result in the activation of Kupffer and hepatic stellate cells. This is accompanied by the release of inflammatory cytokines [34,35] and the infiltration of circulating monocytes and neutrophils [36]. Inducible NO synthase (iNOS) is an isoform of NOS expressed under the influence of stimulation by cytokines or microbial products. Once iNOS is expressed, large amounts of NO are generated in the liver in a sustained fashion, serving as an important regulator and effector during inflammation and infection. In inflamed liver, hepatocytes are situated in an environment where NO is generated from surrounding cells as well as from hepatocytes themselves [37]. A previous study demonstrated that RO decreased iNOS mRNA and protein, as well as nitrite production after ischemia-reperfusion injury in rat hearts [38]. Our data show that rosuvastatin reduced NO (measured as nitrite) level rise induced by BDL. Histopathological findings revealed alterations in liver structure as well as leucocytes infiltration in the BDL group. These inflammatory signs were improved by the administration of RO. Our results are in accordance with previous findings concerning other statins such as fluvastatin and simvastatin where it was proved that these statins, respectively, decreased expression of the transcription nuclear factor kappa beta (activated by inflammation) and reduced hepatic formation of CXC chemokines induced by the BDL model [39,40].

DT-diaphorase or quinone reductase (QR), an enzyme involved in phase II metabolism of xenobiotics, is capable of scavenging superoxide anions generated during oxidative stress and regenerating reduced forms of protective endogenous antioxidant compounds. There is very low expression of QR mRNA and protein in normal human livers, with slightly greater mRNA levels observed in biliary epithelial cells [41,42]. Consequently, it has been suggested that human QR does not play a major role in hepatic xenobiotic metabolism under normal conditions [43]. Instead, QR may be more important during periods of hepatic oxidative stress and damage. QR mRNA protein and activity are markedly increased in mouse liver following bile duct ligation [44,45]. Aleksunes et al. hypothesized that upregulation of QR may represent a common response to liver injury with an oxidative stress component [46]. In accordance with the previous data, our study showed that BDL increased markedly QR activity. Treatment with RO was able to restore it.

In conclusion, we demonstrated that the hydrophilic statin, rosuvastatin, improved biliary obstruction induced-injury, oxidative stress and inflammation in the liver. Our data support the view that statins may represent a promising strategy to be used as adjunct therapy in the case of cholestasis.

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