

Simvastatin decreases hepatic ischaemia/reperfusion-induced liver and lung injury in rats

F. Dibazar¹, B. Hajipour², M.M. Hosseinian³, M.R. Hemmati⁴, A. Ghandiha⁵

¹Departement of Anatomy and Histology, Faculty of Medicine, Islamic Azad University, Tabriz branch, Iran

²Faculty of Medicine, Islamic Azad University, Tabriz branch, Iran

³Departement of Neurology, Faculty of Medicine, Islamic Azad University, Tabriz branch, Iran

⁴Departement of Pathology, Faculty of Medicine, Islamic Azad University, Tabriz branch, Iran

⁵Departement of Pharmacology, Islamic Azad University, Tabriz branch, Iran

[Received 18 May 2008; Accepted 20 July 2008]

Liver failure is still a significant clinical problem after transplantation surgery, tissue resections (the Pringle manoeuvre) and haemorrhagic shock. The restoration of blood flow to an ischaemic region leads to tissue injury at a greater rate than the original ischaemic insult, an event termed "ischaemia-reperfusion injury" (I/R). Despite advances in surgical techniques, I/R still poses a problem of clinical importance. In this research, we studied the effect of simvastatin pretreatment on liver and lung injury induced by hepatic I/R.

Rats were subjected to 30 min of ischaemia followed by 24 h of reperfusion. Simvastatin (10 mg/kg) was administered orally from three days before the operation. After the reperfusion time, serum ALT, AST, LDH and TNF α levels were studied and liver and lung tissues were stained with haematoxylin and eosin and TUNEL to detect apoptotic cells.

Serum aminotransferase activity and LDH and TNF α levels were increased markedly by hepatic I/R, and these were suppressed significantly by simvastatin. The tissue injury index and the number of apoptotic cells via TUNEL staining in the liver and lungs were higher in the I/R group than in the I/R + simvastatin group. These results suggest that simvastatin ameliorates I/R-induced liver and lung tissue damage by inhibiting the level of inflammation and the apoptotic pathways. Simvastatin administration may therefore provide protection against the adverse effects of I/R injury in liver transplantation. (Folia Morphol 2008; 67: 231–235)

Key words: liver, ischaemia/reperfusion, lung, simvastatin

INTRODUCTION

Liver failure is still a significant clinical problem after transplantation surgery, tissue resections (the Pringle manoeuvre) and haemorrhagic shock [10, 17, 19, 30]. The restoration of blood flow to an ischaemic region leads to tissue injury at a greater rate than the original ischaemic insult, an event termed "ischaemia-reperfusion injury" (I/R). Although the

exact mechanisms involved in the pathogenesis of hepatic I/R injury have not been fully elucidated, it is generally believed that activated Kupffer cells release reactive oxygen species, nitric oxide and several proinflammatory cytokines. These cytokines, together with the increased expression of adhesion molecules by sinusoidal endothelial cells, promote liver neutrophil infiltration, thus contributing to the

progression of parenchymal injury [6, 7, 12, 13, 18]. Efforts to attenuate I/R injury have been focused on blocking the events associated with irreversible liver damage. Hepatic I/R, in addition to liver injury, induces remote organ injury, especially in lung tissue [14]. Pharmacological preconditioning includes blocking the injury process, mainly the oxidative and inflammatory pathways, by drug administration.

Statins are a class of compound that competitively inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the first committed step in cholesterol biosynthesis.

Statins can be classified into three categories: naturally derived (lovastatin and pravastatin), semisynthetic (simvastatin), and synthetic (atorvastatin, fluvastatin, cerivastatin, rosuvastatin, and pitavastatin) [22]. It has been shown that statins that contain a lactone structure with high lipophilic properties, that is, simvastatin and lovastatin, exhibit a potent vasodilatory effect in the intact heart [8] and in vessels isolated from various tissues [1, 2, 21, 24, 28]. Increasingly, the pleiotropic properties of statins are being described. In endothelial cells, all of these effects seem to result from the inhibition of cholesterol's precursor, mevalonic acid, which is critical to the isoprenylation of a diverse family of proteins [3, 5]. Simvastatin, a HMG-CoA reductase inhibitor, has been shown to exhibit important immunomodulatory effects independent of lipid lowering [15]. These pleiotropic effects have been demonstrated to include anti-inflammatory actions [27], improvement of endothelial and microvascular function, modulation of endothelial nitric oxide synthase (eNOS) [16], ischaemia/reperfusion [25] and sepsis [23]. However, the effect of statins has not been studied in hepatic I/R injury.

MATERIAL AND METHODS

Animals

Forty-five male Wistar rats were divided into three groups. Group 1, the sham group, underwent laparotomy but did not experience I/R; group 2, the I/R group, underwent ischaemia for 30 min and were reperfused for 24 h; Group 3 underwent ischaemia and reperfusion like group 2 but also received 10 mg/kg simvastatin dissolved in water by oral gavage for three days before the operation.

All the animals had access to water and rat chow *ad libitum* before, during, and after the ischaemia. The animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals

prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Induction of ischaemia

Briefly, the anterior abdominal wall was shaved and prepared with povidone-iodine solution. The abdomen was entered through a midline incision under ether anaesthesia and ischaemia was induced by occluding the blood vessels, including the bile duct, with an atraumatic vascular clamp. As a model for acute experiments, 30 min ischaemia of the liver lobe was performed by clamping the portal vein, hepatic artery and bile duct origin. Reperfusion was initiated by removal of the clamp and the abdomen was closed in two layers with 2-0 silk. Thereafter the abdominal cavity was closed with running sutures and the animals were allowed to awaken without resuscitation by additional fluid. The animals had free access to water and rat chow *ad libitum*. After 24 h of reperfusion the anaesthetised animals were sacrificed and liver and lung tissue and plasma were stored for further examination.

Histological analysis

Excised liver specimens were fixed in 4% PBS-buffered paraformaldehyde and embedded in paraffin. Five-micrometre liver sections stained with haematoxylin and eosin were evaluated at $\times 200$ magnification by a point-counting method for the severity of hepatic injury.

In situ detection of apoptotic cells

Serial sections of 4- μ m thickness were prepared. TUNEL staining was performed with the use of an *in situ* apoptosis detection kit according to the manufacturer's instruction (Boehringer Mannheim, Germany) and examined by light microscopy. The apoptotic index (AI) was calculated as the percentage of stained cells, namely: $AI = \text{number of apoptotic cells} / 100 / \text{total number of nucleated cells}$.

The histopathological scoring analysis was performed according to previously described methods with modifications. For liver and lungs the assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate) to 3 (severe) for each of the following six parameters from the liver sections: cytoplasmatic colour fading, vacuolisation, nuclear condensation, nuclear fragmentation, nuclear fading and erythrocyte stasis, or for six parameters from the lung sections: interstitial oedema, alveolar oedema, alveolar

Table 1. Serum activities of ALT, AST, LDH and TNF α measured in rats 24 h after reperfusion. Results expressed as mean \pm SEM

	Sham	I/R	I/R + simvastatin
ALT [U/L]	58 \pm 10.3	135 \pm 14.1	87 \pm 13.3
AST [U/L]	152 \pm 15.8	335 \pm 17.1	231 \pm 15.4
LDH [U/L]	1391 \pm 172	4558 \pm 210	3317 \pm 183
TNF α [pg/g tissue]	5.1 \pm 1.3	64.7 \pm 5.2	41.4 \pm 4.1

Table 2. Tissue injury index of the liver and lungs and the number of apoptotic cells 24 h after reperfusion

	Sham	I/R	I/R + simvastatin
Liver injury index	1.1 \pm 0.3	18.6 \pm 3.6	8.3 \pm 2.7
Liver apoptosis	1 \pm 0.2	33.4 \pm 5.2	18.1 \pm 3.4
Lung injury index	0.8 \pm 0.3	16.9 \pm 3.1	10.3 \pm 2.4
Lung apoptosis	1.3 \pm 0.2	40.4 \pm 8.3	21.3 \pm 6.2

haemorrhage, atelectasis, inflammatory cellular infiltration and pulmonary congestion [9, 32].

Biochemical analysis

Blood samples were collected. Serum was obtained by centrifugation. Samples were stored at -20°C until analysis. TNF α , ALT, AST, and LDH levels were determined by commercial kits.

Statistical analysis

Mean standard error of the mean (SEM) values for various groups were compared using Fisher's exact test, analysis of variance (ANOVA) or ANOVA on ranks, as appropriate, with $p < 0.05$ selected prior to the study as the criterion for significance of differences between groups.

RESULTS

Liver function

We examined the serum activities of AST and ALT as markers of liver function at 24 h after I/R. The activities of AST, ALT and LDH in the I/R group were markedly increased, compared with the SHAM or I/R + simvastatin group (Table 1).

Tumour necrosis factor α

The level of TNF α was increased in the I/R group. This increase was significantly diminished by prior simvastatin treatment in the I/R + simvastatin group (Table 1).

Analysis by the TUNEL method of apoptosis in the liver and lung tissue showed that the apoptosis index was significantly lower in the simvastatin + I/R group than in the I/R group. Very few TUNEL-positive hepatocytes were detected in the sham groups (Table 2).

Tissue morphology

In the liver and lung tissue no significant changes were found in the sham group at light microscopy. At 24 h after reperfusion extensive karyopycnosis, severe necrosis and loss of intercellular borders were seen in the I/R group. At these time points hepatocyte damage was slighter in the simvastatin treatment group than in the I/R group (Table 2). These results indicated that hepatic injury induced by I/R was much more severe in the I/R group than in the C + I/R group. Moreover, in the lungs tissue injury, including interstitial oedema, alveolar oedema, alveolar haemorrhage, atelectasis, inflammatory cellular infiltration and pulmonary congestion, in the I/R group was significantly more severe than in the I/R + simvastatin group (Table 2).

DISCUSSION

In this study, we have demonstrated that simvastatin pretreatment afforded functional and histologic hepatic and lung protection in hepatic I/R induced injury in rats, and this was accompanied by suppression of TNF α production and subsequent

inflammation, hepatic and lung tissue necrosis and apoptosis.

Although liver I/R is more commonly induced by occluding the hepatic artery and portal vein to the left and median liver lobes (60–70% partial liver ischaemia) in animal models according to previously published reports, temporary occlusion of the hepatoduodenal ligament (the Pringle manoeuvre) is more often used in major liver surgery, including liver transplantation [1]. In the present study we mimicked this model, in which hepatic I/R injury induced by totally occluding the portal vein and hepatic artery caused more serious damage to livers than the partial liver ischaemia in our other report [32], supported by the comparison of the activities of AST and ALT, and the histological alterations [11]. The more severe damage to the liver in turn produces and releases more destructive proinflammatory cytokines into the circulation, such as TNF α and oxygen-derived radicals, causing subsequent damage to other organs, including the kidney, lung, and intestine [14].

In the liver I/R induces inflammatory mediators such as chemokines, cytokines, and adhesion molecules. These mediators are thought to initiate the inflammatory cascade that leads to leukocyte recruitment and microcirculatory compromise, with resultant liver dysfunction.

Hepatocyte cells that are lethally injured after ischaemic or toxic insults can die by necrosis or apoptosis. Contrary to apoptosis, which is a well coordinated process, necrosis usually results from overwhelming cellular ATP depletion that is beyond repair, and has been known to be an unregulated event. However, recent studies suggest that necrosis is also controlled by a specific programme that involves many signalling cascades such as reactive oxygen species and stress kinases [31].

Statins reduce cell adhesion by blocking expression of monocyte chemoattractant protein-1 [4]. They also inhibit both expression and activation of integrin, which is essential for leukocyte adhesion [20]. Other research has indicated that statins interfere with the expression of intercellular adhesion molecule-1 (ICAM-1) and cytokines in endothelial cells and monocytes [26]. Another consequence of the inhibition of geranylgeranylation of *Rho* GTPase by statins is increased expression and activity of eNOS. The resultant increase in nitric oxide production causes reduced expression of adhesion molecules, including selectins and ICAM-1 [29]. The above-mentioned anti-inflammatory mechanisms of statins are likely

to be responsible for the protective effect of simvastatin that we observed in the inhibition of TNF α production in this rat model of hepatic I/R.

In summary, simvastatin pretreatment protects the liver and lungs from warm hepatic I/R injury through multiple pathways, and inhibits cell apoptosis, decreased cellular necrosis and TNF α production.

REFERENCES

1. Alvarez De Sotomayor M, Herrera MD, Marhuenda E, Andriantsitohaina R (2000) Characterization of endothelial factors involved in the vasodilatory effect of simvastatin in aorta and small mesenteric artery of the rat. *Br J Pharmacol*, 131: 1179–1187.
2. Bravo L, Herrera MD, Marhuenda E, Perez-Guerrero C (1998) Cardiovascular effects of lovastatin in normotensive and spontaneously hypertensive rats. *Gen Pharmacol*, 30: 331–336.
3. Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F (1999) New insights into the pharmacodynamic and pharmacokinetic properties of statins. *Pharmacol Ther*, 84: 413–428.
4. Diomedea L, Albani D, Sottocorno M, Donati MB, Bianchi M, Fruscella P, Salmona M (2001) In vivo anti-inflammatory effect of statins is mediated by nonsterol mevalonate products. *Arterioscler Thromb Vasc Biol*, 21: 1327–1332.
5. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. *Nature*, 1990; 343: 425–430.
6. Gonzalez-Flecha B, Cutrin JC, Boveris A (1993) Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest*, 91: 456–464.
7. Grattagliano I, Vendemiale G, Lauterburg BH (1999) Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. *J Surg Res*, 86: 2–8.
8. Gryglewski RJ, Uracz W, Swies J, Chlopicki S, Marcinkiewicz E, Lomnicka M (2001) Comparison of endothelial pleiotropic actions of angiotensin converting enzyme inhibitors and statins. *Ann NY Acad Sci*, 947: 229–245.
9. Heijnen BHM, Straatsburg IH, Gouma DJ, van Gulik TM (2003) Decrease in core liver temperature with 10°C by in situ hypothermic perfusion under total hepatic vascular exclusion reduces liver ischemia and reperfusion injury during partial hepatectomy in pigs. *Surgery*, 134: 806–817.
10. Huguet C, Gavelli A, Bona S (1994) Hepatic resection with ischemia of the liver exceeding one hour. *J Am Coll Surg*, 178: 454–458.
11. Ito K, Ozasa H, Horikawa S (2005) Effects of prior splenectomy on remnant liver after partial hepatectomy with Pringle maneuver in rats. *Liver Int*, 25: 438–444.
12. Jaeschke H, Farhood A (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver in vivo. *Am J Physiol Gastrointest Liver Physiol*, 260: G355–G362.
13. Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ (1991) Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. *Free Radic Res Commun*, 15: 277–284.

14. Jiang H, Meng F, Li W, Tong L, Qiao H, Sun X (2007) Splenectomy ameliorates acute multiple organ damage induced by liver warm ischemia reperfusion in rats. *Surgery*, 141: 32–40.
15. Kwak B, Mulhaupt F, Myit S (2000) Statins as a newly recognized type of immunomodulator. *Nat Med*, 6: 1399–1402.
16. Laufs U, Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem*, 273: 24266–24271.
17. Lemasters JJ, Thurman RG (1997) Reperfusion injury after liver preservation for transplantation. *Annu Rev Pharmacol Toxicol*, 37: 327–338.
18. Li JM, Shah AM (2001) Differential NADPH versus NADH dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase. *Cardiovasc Res*, 52: 477–486.
19. Liu DL, Jeppsson B, Hakansson CH, Odselius R (1996) Multiple-system organ damage resulting from prolonged hepatic inflow interruption. *Arch Surg*, 131: 442–447.
20. Liu L, Moesner P, Kovach NL, Bailey R, Hamilton AD, Sebti SM, Harlan JM (1999) Integrin-dependent leukocyte adhesion involves geranylgeranylated protein(s). *J Biol Chem*, 274: 33334–33340.
21. Lorkowska B, Chlopicki S (2005) Statins as coronary vasodilators in isolated bovine coronary arteries: involvement of PGI₂ and NO. *Prostaglandins Leukot Essent Fatty Acids*, 72: 133–138.
22. Manzoni M, Rollini M (2002) Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl Microbiol Biotechnol*, 58: 555–564.
23. Merx MW, Liehn EA, Janssens U, Lütticken R, Schrader J, Hanrath P, Weber C (2004) HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis. *Circulation*, 109: 2560–2565.
24. Mraiche F, Cena J, Das D, Vollrath B (2005) Effects of statins on vascular function of endothelin-1. *Br J Pharmacol*, 144: 715–726.
25. Naidu BV, Woolley SM, Farivar AS, Thomas R, Fraga C, Mulligan MS (2003) Simvastatin ameliorates injury in an experimental model of lung ischemia-reperfusion. *J Thorac Cardiovasc Surg*, 126: 482–489.
26. Niwa S, Totsuka T, Hayashi S (1996) Inhibitory effect of fluvastatin, an HMG-CoA reductase inhibitor, on the expression of adhesion molecules on human monocyte cell line. *Int J Immunopharmacol*, 18: 669–675.
27. Pruefer D, Makowski J, Schnell M (2003) Simvastatin inhibits inflammatory properties of *Staphylococcus aureus* alpha toxin. *Circulation*, 106: 2104–2110.
28. Tesfamariam B, Frohlich BH, Gregg RE (1999) Differential effects of pravastatin, simvastatin, and atorvastatin on Ca²⁺ release and vascular reactivity. *J Cardiovasc Pharmacol*, 34: 95–101.
29. Tsiara S, Elisaf M, Mikhailidis DP (2003) Early vascular benefits of statin therapy. *Curr Med Res Opin*, 19: 540–556.
30. Vedder NB, Fouty BW, Winn RK, Harlan JM, Rice CL (1989) Role of neutrophils in generalized reperfusion injury associated with resuscitation from shock. *Surgery*, 106: 509–516.
31. Yaglom JA, Ekhterae D, Gabai VL, Sherman MY (2003) Regulation of necrosis of H9c2 myogenic cells upon transient energy deprivation. Rapid deenergization of mitochondria precedes necrosis and is controlled by reactive oxygen species, stress kinase JNK, HSP72 and ARC. *J Biol Chem*, 278: 50483–50496.
32. Zegdi R, Fabre O, Cambillau M, Fornès P, Tazi KA, Shen M, Hervé P, Carpentier A, Fabiani JN (2003) Exhaled nitric oxide and acute lung injury in a rat model of extracorporeal circulation. *Shock*, 20: 569–574.