Modulation of Rat Liver Regeneration after Partial Hepatectomy by Dietary Cholesterol

Pavel Živný, Helena Živná, Vladimír Palička, Lenka Žaloudková, Petra Mocková, Jolana Cermanová, Stanislav Mičuda

ABSTRACT
Introduction: The aim of study was to evaluate impact of long-term dietary cholesterol overload on the cholesterol homeostasis and liver regeneration.

Material and Methods: Serum lipid parameters, 14C-cholesterol incorporation, liver DNA synthesis and protein expression was determined in partially hepatectomized (PH) rats fed with a standard (SLD) or hypercholesterolemic (CHOL) diet.

Results: 29-day intake of CHOL diet before PH produced increase in serum total cholesterol, LDL lipoprotein, and triglyceride concentration. PH provoked decrease in serum total cholesterol and triglyceride concentration in both groups. PH was associated with increase in serum ALT activity more pronounced in CHOL animals. Hepatic DNA synthesis was increased after PH in both groups, but lower in CHOL. Hypercholesterolemic diet reduced the absorption of radiolabelled cholesterol in intestine and then activity in blood and liver. The 14C-cholesterol hepatic activities tend to increase after PH in both groups. CHOL diet produced up-regulation of Acyl-CoA:cholesterol acyltransferase-2 protein expression. PH was associated with increase of LDL receptor and Acyl-CoA:cholesterol acyltransferase-2 protein expression in both dietary groups.

Discussion: Liver regeneration after PH is negatively influenced by CHOL diet. The increased uptake of cholesterol in the liver after PH associated with up-regulation of LDL receptor protein expression suggests preferential use of extrahepatic cholesterol by the liver.

KEYWORDS
liver; partial hepatectomy; cholesterol; LDL receptor; ACAT-2; rat

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INTRODUCTION

Hypercholesterolemia has been considered as a major risk factor for cardiovascular disease such as coronary heart disease and atherosclerosis (1), a leading cause of death and disability in developed countries. Moreover the role of cholesterol in the pathogenesis of non-alcoholic steatohepatitis (NASH) remains unclear. Results of Zhu et al. suggested that cholesterol markedly promoted apoptosis of steatotic HepG2 cells in vitro, likely through the up-regulation of Bax and caspase-3 expression. Other authors suggested that abnormal lipid metabolism may contribute to the increase of reactive oxygen species (ROS) and inflammation and induced NASH (2, 3).

Liver is the central organ in the regulation of whole-body cholesterol homeostasis. The role in the cholesterol metabolism is multiplex and includes the synthesis of cholesterol and cholesterol-carrying apoproteins; catabolism of cholesterol to bile acids; receptor-mediated clearance of cholesterol containing lipoproteins; and esterification of cholesterol (4, 5). Not surprisingly, high cholesterol dietary intake induces changes of these processes and invoke suppression of cholesterol de novo synthesis and increased storage of lipids in the liver (2, 6). In addition, long-term hypercholesterolemic diet is associated with potentiated ed signalling by adhesion molecules and activation of fat-storing cells to become fibrogenic effector leading to liver fibrosis (7, 8).

Partial hepatectomy (PH) in rats is widely used model to study liver regeneration, which is accompanied by cell division and by outstanding cell and organelle membrane production. This process requires quantity of cholesterol (9). Hepatocytes after PH proliferate synchronously, they enter in G1 phase and begin to synthesize DNA 18 h after PH and divide after 24 h. It has been shown that cholesterol is necessary in early G1 phase during cell duplication. During liver regeneration, an increase in chromatin cholesterol is observed between 6 and 18 h after hepatectomy (7). Therefore, appropriate cholesterol intake and balanced diet is essential for liver regeneration (9).

The aim of present study was (1) to evaluate impact of long-term dietary cholesterol overload on the cholesterol homeostasis and then (2) to evaluate the influence of this status on liver regeneration induced by surgical removal of 70% of liver parenchyma in rats. To obtain relevant information, changes in serum biochemical parameters, hepatic DNA synthesis, cholesterol content in the main organs of cholesterol turnover and hepatic expression of key enzymes of cholesterol metabolism were evaluated in these animals.

MATERIALS AND METHODS

PREPARATION OF LABORATORY DIET

Diet was prepared according to available literature (www.testdiet.com, www.dyets.com) from casein (PML Inc., Novy Bydžov, CZ), cornstarch (Skrobarny Pelhrimov, CZ), cellulose (Phrikolat, Chemische Erzeugnisse GmbH, Germany), choline chloride, L-cysteine, L-arginine and sucrose (Fisher Scientific, Ltd., Pardubice, CZ), corn oil (CANO Ltd. Hermanuv Mestec, CZ), DL-methionine (Sigma-Aldrich, Ltd. Prague, CZ), mixture of vitamins and minerals according to AIN-93, TestDiet.

The half of the prepared diet was control diet (SLD). Second half of diet was enriched by cholesterol to the final concentration of 4% (CHOL). These diets were fabricated into pellets and dried in 60 °C in food dryer.

ANIMALS

The Ethical Expert Committee of Medical Faculty, Charles University, Hradec Králové approved the experiment protocol (No. 20287/2005-30/300). All operations were performed in total ether anesthesia. Male Wistar rats (Biotest Inc., Konarovice, Czech Republic) were placed in plastic cages under standard conditions. They were in standard room temperature 22 ± 2 °C, twelve hours light/dark system, air humidity 30–70%. The rats (8 weeks old) were randomly divided into 2 groups, 24 rats each with starting body weight 256 ± 15 g. They were fed with above mentioned diets and drank tap water ad libitum for 29 days. 1st group: (SLD), were fed with standard laboratory diet, and 2nd group: (CHOL), were fed with cholesterol enriched diet (80–100 mg of cholesterol/rat/day). Eight rats from each group were sacrificed on 29th day of experiment (SLD-int, CHOL-int), others (8 + 8) underwent at the day 28 two third partial hepatectomy (PH) and then were sacrificed 18 hours (8 + 8 rats, SLD-18, CHOL-18), and 24 hours (8 + 8 rats, SLD-24, CHOL-24) after partial hepatectomy (10).

“C-cholesterol (37 kBg/100 g of body weight, APCzech, Prague, CZ) was administered p.o. 24 hours before sacrifice to intact rats or immediately after PH. The 3H-thymidin (740 kBq/100 g of body weight, Lacomed Ltd., Řez u Prahy, CZ) was administered i.v. 1 hour before sacrifice by exsanguination from abdominal aorta in all rats.

The liver was removed and immediately frozen in liquid nitrogen and stored at ~80 °C until analyses.

ANALYSES

Serum total cholesterol, triglyceride (TAG) concentration and ALT activity (ukat/l) was measured by automated enzymatic methods on Modular Roche analyser (Roche, Mannheim, Germany). Liver cholesterol concentration was determined using colorimetric kits (Lachema, Brno, CZ).
The activity of ¹⁴C-cholesterol (Bq/g of tissue or ml of blood) was determined after ethanol-acetone extraction and saponification and radioactivity was quantitated by scintillation counting (11). Newly synthesized bile acids with incorporated ¹⁴C-cholesterol in liver were assessed after ethanol extraction, precipitation by digitonin (10) and radioactivity was quantitated by scintillation counting. Liver DNA synthesis was determined by incorporation of methyl-¹³⁵H-thymidine to liver DNA (11). The radioactivity of the samples was measured by liquid scintillation system on Beckman Coulter LS 6000LL (Fullerton, CA, USA). The liver DNA content was determined with diphenylamine reagent (12).

WESTERN BLOT

Hepatic expression of key enzymes of cholesterol metabolism, namely 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, low-density lipoprotein (LDL) receptor, and acyl-coenzyme A:cholesterol acyltransferase (ACAT-2) was assessed by Western blot.

Membrane preparation. Samples were prepared as described previously (13). Briefly, livers were minced in ice-cold Tris-sucrose buffer containing 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 2 μg/mL aprotinin, 50 μg/mL benzamidine, and 40 μg/mL phenylmethylsulfonyl fluoride (PMSF) and homogenized. A membrane-enriched microsomal pellet was obtained from the postnuclear supernatant after a 100,000 g ultracentrifugation at 4 °C for 60 minutes. Acquired pellet was resuspended in Tris-HCl buffer. The protein concentration was determined with the BCA assay (Pierce, Rockford, IL, USA) and samples were stored at −80 °C.

Immunoblot analysis. Crude membrane-containing homogenates (50 μg) from freshly harvested livers were incubated with sample buffer at room temperature for 30 minutes and separated on a 7.5% polyacrylamide gel. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), it was blocked for 30 minutes and separated on a 7.5% polyacrylamide gel. After washing five times with TTBS, the membranes were incubated with sample buffer at room temperature for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and incubated with primary antibodies (1:500) for 1 h, washed, and incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1,000). After washing five times with TTBS, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare, Prague, CZ) and subjected to autoluminography for 1–5 min. The immunoreactive bands on the exposed films were scanned with densitometer ScanMaker i900 (UMAX, Prague, CZ) and semi-quantified using the QuantityOne imaging software (Bio-Rad).

HISTOLOGY

Liver tissue for histopathological examination was obtained from one standard site (processus anterior dexter et processus caudatus lobi caudati) and fixed in 10% buffered formalin. The 3 μm paraffin sections were stained with hematoxylin–eosin. Steatosis was graded by semi-quantitative analysis as follows: mild = 5–29%; moderate = 30–59%; and severe = more than 60% of hepatocytes affected. Each biopsy was analyzed and graded by the same pathologist who was blinded to the rats group.

RESULTS

The results of serum biochemical analyses are summarized in Table 1. 29-day continual intake of high cholesterol diet in rats before PH produced 35% and 136% increase in serum total cholesterol and triglyceride. PH induced insignificant decrease in serum total cholesterol and triglyceride concentration in both SLD and CHOL groups. Alanine aminotransferase activities were significantly higher after PH in both dietary groups in comparison with intact rats, but changes were more pronounced in CHOL group.

Liver regenerative mechanism as evaluated by DNA synthesis and total content of DNA together with cholesterol content in the liver and changes in liver weights are listed in Table 2. PH in control diet fed animals produced increase in DNA synthesis in both SLD-PH groups with progression according to duration of PH. Total hepatic content of DNA and cholesterol was not changed in SLD-PH animals when compared with SLD-int. The rats of CHOL-int group had similar synthesis of DNA as SLD-int group. Nevertheless, PH in these animals produced significantly lower liver DNA synthesis 18 and 24 hours after surgical procedure together with decreased total content of liver DNA 24 hour after PH in comparison with respective SLD-PH rats. The liver weight in CHOL-int rats was significantly higher than in SLD-int, but smaller when compared with respective 24 hour PH groups. Liver cholesterol content was significantly higher in all CHOL rats comparing to corresponding SLD group. In comparison to respective intact groups, hepatic cholesterol content in both diet groups slightly decreased during 18 hour after PH.

To evaluate actual turnover of cholesterol in blood, liver and intestine, ¹⁴C cholesterol was applied p.o. to all rats 18 or 24 hour before being sacrificed. The results of incorporation of radiolabeled cholesterol in tissues are presented in Table 3. While PH in SLD animals did not influence the activities of cholesterol in intestine, or blood, the CHOL-int animals exerted significantly lower ¹⁴C-cholesterol activities in all three evaluated compartments when compared with SLD-int group. The ¹⁴C-cholesterol hepatic activities tend to increase after PH in both diet groups when compared with corresponding intact group.

To study expression of crucial receptors and enzymes involved in hepatic turnover of cholesterol we performed western blot analysis of HMG-CoA reductase, LDL receptor, and ACAT-2. Results are presented in Fig. 1–3. The HMG-CoA reductase protein expression was similar in intact animals of both diet groups. In addition, correspond-
ing progression of changes was detected in all groups after PH. We observed insignificant decrease of HMG-CoA reductase 18 hours after PH and then increase 24 hours after PH (Fig. 1).

The expression of ACAT-2 was slightly induced in intact cholesterol fed rats (Fig. 2). The SLD rats had significantly elevated ACAT-2 expression especially 18 hour after PH in comparison with SLD intact rats. Similarly, the rats of CHOL group had significant elevation of ACAT-2 expression after both intervals of PH.

The rats in CHOL group had insignificantly lower LDL receptor protein expression before PH versus SLD rats (Fig. 3). The significant increase in LDL receptor protein expression was observed in both dietary groups 18 hours and 24 hours after PH with changes being more intensive in CHOL rats.

Macroscopic and microscopic examination of the liver of SLD and CHOL animal is presented in Fig. 4–7.

Liver of a rat fed by SLD diet shows normal macroscopic finding (Fig. 4).

The liver tissue of a control rat shows no histopathologic changes (Fig. 5).

The liver of a CHOL rat shows macroscopic steatosis (Fig. 6).

The liver after a 29 days CHOL diet shows lobular microvesicular steatosis (>30%), no inflammatory cells were observed (Fig. 7).

DISCUSSION

In the present study, we demonstrated significant changes in cholesterol homeostasis after long-term hypercholesterolemic diet in rats which were associated with impaired liver regeneration. High cholesterol intake induced increased serum total cholesterol and TAG concentrations.

Tab. 1 Serum concentrations of total cholesterol, triglycerides (mmol/l) and alanine-aminotransferase activities (μkat/l).

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<tr>
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<th>SLD</th>
<th>CHOL</th>
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<tbody>
<tr>
<td></td>
<td>SLD-int</td>
<td>SLD-18</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.3 ± 0.3</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>TAG</td>
<td>1.4 ± 0.2</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>ALT</td>
<td>0.7 ± 0.09</td>
<td>6.8 ± 1.0</td>
</tr>
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</table>

Values are means ± SEM (n = 8).
* P < 0.05, ** P < 0.01, *** P < 0.001 all groups vs. SLD-intact animals;
† P < 0.05, †† P < 0.001 PH group vs. corresponding intact group (SLD or CHOL);
‡ P < 0.05 respective CHOL-PH group vs. corresponding SLD-PH group

Tab. 2 Specific activity (s.a. – synthesis) of liver DNA (Bq/μg DNA), total content (t.c.) of liver DNA (μg × 10³/g jater), weight of liver remnant (g/100 g of body weight) and liver cholesterol concentration (μg/g).

<table>
<thead>
<tr>
<th></th>
<th>SLD</th>
<th>CHOL</th>
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<tr>
<td></td>
<td>SLD-int</td>
<td>SLD-18</td>
</tr>
<tr>
<td>s.a. DNA</td>
<td>0.2 ± 0.03</td>
<td>2.3 ± 0.44</td>
</tr>
<tr>
<td>t.c. DNA</td>
<td>1.7 ± 0.04</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Liver weight</td>
<td>3.5 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Liver cholesterol</td>
<td>4.3 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 8).
* P < 0.05, ** P < 0.01, *** P < 0.001 all groups vs. SLD-intact animals;
† P < 0.05, †† P < 0.001 PH group vs. corresponding intact group (SLD or CHOL);
‡ P < 0.05 respective CHOL-PH group vs. corresponding SLD-PH group;
xxx P < 0.001 SLD-18 vs. SLD-24 and CHOL-18 vs. CHOL-24

Tab. 3 14C-cholesterol activities in selected tissues, in blood and as bile acid in liver tissue (Bq/g of tissue, Bq/ml of blood).

<table>
<thead>
<tr>
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<th>SLD</th>
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<tr>
<td></td>
<td>SLD-int</td>
<td>SLD-18</td>
</tr>
<tr>
<td>Intestine</td>
<td>1952 ± 797</td>
<td>1948 ± 473</td>
</tr>
<tr>
<td>Blood</td>
<td>616 ± 44</td>
<td>322 ± 70</td>
</tr>
<tr>
<td>Liver</td>
<td>833 ± 123</td>
<td>1467 ± 235</td>
</tr>
<tr>
<td>Bile acid in liver tissue</td>
<td>71.2 ± 15.1</td>
<td>74.3 ± 10.9</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 8).
* P < 0.05, ** P < 0.01, *** P < 0.001 all groups vs. SLD-intact animals
CHOL rats developed steatosis, which was proven macroscopically and microscopically. PH in these animals lead to lower hepatic DNA synthesis, DNA content and cholesterol uptake and increased ALT activity in comparison to intact animals which indicate problems in liver reparative processes. In addition, we observed induction of LDL receptor protein expression and increased uptake of cholesterol by the liver after PH.

The high-cholesterol diet consumption reduced cholesterol absorption from intestine in mice (14), in rats (15), and probably in humans (16). The assumed mechanism is decrease of intestinal cholesterol absorption by up-regulation of adenosine triphosphate-binding cassette cholesterol transporter – Abcg5, Abcg8 (15) or a Niemann-Pick CI Like 1 (NPC1L1) protein localized in jejunal enterocytes, which are critical for intestinal cholesterol absorption (17). These data comply with the results of our study where we demonstrated that the rats of CHOL group had inhibited exogenous cholesterol absorption, and 14C-cholesterol was increased in faeces in comparison with SLD group (unpublished observation). This fact is agreed by significant decrease of 14C cholesterol activities in intestinal wall tissues in CHOL groups (and consequently in blood, liver and intrahepatic bile acid).

We observed slight increase in hepatic ACAT-2, and unchanged LDL-receptor and HMG-CoA reductase protein expression between SLD and CHOL rats before PH. Spectrum of studies dealing with these protein expressions after hypercholesterolemic diet brought controversial results. Our results are in agreement with some of these reports pointing to induction of ACAT-2 and minimal
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changes of HMG-CoA reductase and LDL receptor during long-term cholesterol overload (5, 6, 18). One explanation for minimal changes in respective hepatic proteins observed in our study might be the decreased intestinal absorption of cholesterol, which reduces influence of dietary cholesterol on the liver proteins.

Liver regeneration after PH is accompanied by cell division and (cell and organelle) membrane production is a process requiring cholesterol (7). The rats after PH lost about 67–70% of liver parenchyma together with cholesterol synthesis enzymes and cholesterol store, nevertheless they have to induce regenerative processes. As a consequence, serum cholesterol concentration decreased after PH in both groups, which comply with our previous results (19). Similarly in humans, hypocholesterolaemia is a frequent and typical phenomenon of many acute situations in the critically ill (20). Albi et al. (9) proved that hepatic cholesterol increases during liver regeneration after PH, first as a linked fraction and then, when DNA synthesis starts, as a free fraction. Herein, we demonstrated that regenerating liver tissue in rats after PH gained preferentially cholesterol from circulation. This concept is supported by:

1. Increased content of 14C-cholesterol in the liver after PH indicating increased uptake from blood. This effect is most probably consequence of observed up-regulation of liver LDL receptor expression after PH. It suggests the effort of remnant hepatocytes to use LDL cholesterol, mainly in rats of CHOL groups which is in accordance with the results of other authors (21, 22). In agreement, Bocchetta et al. (23) demonstrated an induction of LDL receptor gene expression shortly (2–4 h) after PH.

2. Up-regulation of liver ACAT-2 synthesis for esterification of (newly) absorbed liver cholesterol. Our results show, that ACAT-2 expression increased significantly in all PH groups indicating activation of regenerative processes such membrane component synthesis.
3. Stable expression of HMG-CoA reductase after PH, with only an insignificant decrease 18 hour after PH and slight increase 24 hours after PH. Similarly, Cheng et al. (24) demonstrated that cholesterol synthesis was selectively decreased 24 hours after PH in rat hepatocytes. Bakalar et al. (25) proved decrease of cholesterol precursor synthesis as a major cause of hypercholesterolemia in the critically ill patients with multiple trauma.

We evaluated DNA synthesis as the marker of liver regeneration. After PH in SLD animals we observed steady increase in \(^{3}H\)-thymidine incorporation indicating liver regeneration. This process was markedly delayed in CHOL group. We suppose that physiological turnover of adequate endogenous and exogenous cholesterol makes better conditions for triglyceride metabolism with early onset and advantageous course of liver regeneration. Blaha et al. (26) showed necessity of lipids for liver regeneration after PH which was significantly better in balanced enteral feeding in comparison with overfeeding. The rats fed by high cholesterol diet had blocked the cholesterol absorption from intestine, and endogenous synthesis of cholesterol is not flexible enough (24, 25), which might be followed by deceleration of DNA liver synthesis.

The negative effect of high cholesterol diet demonstrated by the course of liver regeneration was expressed among others also by higher transaminase activities in CHOL groups (especially 18 hours after PH). The reason of this fact is not completely resolved. It may be problem originating from higher membrane fluidity (8), severe steatosis (2), or impaired ACAT-2-mediated protection of hepatocytes against free cholesterol toxicity (27).

High cholesterol diet consumption in CHOL group leads to status with higher stores of cholesterol in the liver. The rats of CHOL group had liver steatosis, in spite of that both diets (SLD and CHOL) had an identical content of non-steroid lipids (5% oil of corn). Therefore, the liver steatosis was the result of abundant intake of exogenous cholesterol only, and subsequent different triglyceride distribution. This is in agreement with previously reported rapid deposition of lipid droplets in the liver during a high cholesterol diet (2). The lipid accumulation in the liver results from an imbalance between hepatic fatty acid inflow, triglyceride synthesis and excretion (3).

In conclusion, the liver regeneration in rats after PH is negatively influenced by high cholesterol diet. PH produced preferential use of extrahepatic cholesterol by the liver through LDL receptor up-regulation. However, the uptake of cholesterol to liver was less effective in CHOL rats after PH following delayed liver regeneration.

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