Age-induced hypercholesterolemia in the rat relates to reduced elimination but not increased intestinal absorption of cholesterol

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Karolinska Institute at 1Center for Endocrinology, Metabolism, and Diabetes, Department of Medicine and Molecular Nutrition Unit, Center for Nutrition and Toxicology, NOVUM; and 2Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska University Hospital, Huddinge, Stockholm, Sweden

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Gálman C, Matasconi M, Persson L, Parini P, Angelin B, Rudling M. Age-induced hypercholesterolemia in the rat relates to reduced elimination but not increased intestinal absorption of cholesterol. Am J Physiol Endocrinol Metab 293:E737–E742, 2007. First published June 19, 2007; doi:10.1152/ajpendo.00166.2007.—Plasma cholesterol increases in normal aging in both rodents and humans. This is associated with reduced elimination of cholesterol as bile acids (BAs) and decreased receptor-mediated clearance of plasma LDL, changes that can be reversed by treatment with growth hormone (GH). The level of intestinal absorption of cholesterol may also contribute to the development of hypercholesterolemia. In this study, we investigated whether cholesterol absorption increases with age and whether any such age-related change could be influenced by treatment with GH or ezetimibe (EZE). Male rats aged 6 and 18 mo were studied with and without GH or EZE treatment. BA synthesis was reduced and plasma cholesterol was increased in the old animals, whereas cholesterol absorption was unaltered. Cholesterol absorption was not altered by GH treatment but was reduced by EZE in both groups of animals. Hepatic LDL receptors (LDLRs), scavenger receptor class B type 1, and proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) transcripts were unchanged in old animals. GH treatment induced LDLRs, PCSK9 transcripts, and BA synthesis. We conclude that the age-induced hypercholesterolemia in the rat and its reversal by GH treatment relates to altered degradation of cholesterol in the liver and is not due to changes in cholesterol absorption.

aging; ezetimibe; hyperlipidemia; cholesterol absorption; growth hormone

A number of metabolic changes occur with normal aging in both animals and humans (9). Among these, phenomena such as reduced physical activity, decreased oxygen consumption, redistribution of body tissues with a relative increase in adipose over muscle mass, decreased insulin sensitivity, and increased blood pressure may all contribute to the acceleration of atherosclerosis known to occur with age. Of particular interest is the fact that plasma levels of total and LDL-cholesterol are well known to increase with normal aging (11, 12, 18). Elevated plasma LDL-cholesterol levels represent one of the key causal factors for the development of atherosclerosis and subsequent coronary heart disease (CHD) and are in fact a prerequisite for other risk factors such as smoking, diabetes, inflammation, and hypertension to be active. The important role of plasma LDL-cholesterol for the development of arteriosclerosis was recently highlighted from studies on subjects with genetically caused reductions of LDL-cholesterol due to variations in the gene for proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9), an enzyme that degrades the LDL receptor (LDLR) (22). Strikingly, as little as 15% reduction of plasma LDL-cholesterol, within the “normal levels” for plasma LDL-cholesterol, during a follow-up time of 15 yr showed that the risk to develop CHD was lowered by 47% (7). Cholesterol metabolism is profoundly modified during normal aging (6), and in humans plasma LDL-cholesterol increases by about 40% from 20 to 60 yr of age (46). Thus, due to the magnitude of change with age, it is likely that the age-dependent increase in plasma LDL-cholesterol in part contributes to the concomitant enhanced incidence of CHD, although there are also other factors that may contribute (5). The physiological mechanism for this age-dependent change in plasma cholesterol is still unclear. It is therefore of importance to understand the physiological mechanisms for why plasma cholesterol increases with age. Plasma cholesterol also increases with age in rodents (33). The level of plasma LDL is determined by the balance between LDL synthesis and elimination. In humans as well as in rats, the plasma clearance of LDL is reduced with age (12, 14, 18), probably reflecting a reduced number of hepatic LDLRs observed in rats (14). This may in turn be related to the reduced breakdown of cholesterol to bile acids (BAs), which occurs in rodents with age (33, 44). In rats and mice, it has been reported that intestinal cholesterol absorption may increase with age (20, 24, 44). However, data are not fully uniform on this matter, which may be due to strain and species differences as well as to the assay used to evaluate cholesterol absorption.

During aging, the secretion of growth hormone (GH) is progressively reduced (9, 42). Interestingly, GH exerts several important effects on lipid metabolism in adult humans and animals (4, 15, 25). We have previously shown that GH has important regulatory effects on several steps in cholesterol metabolism. Thus, the expression of hepatic LDLRs and the enzymatic activity of cholesterol 7α-hydroxylase (Cyp7a1) are both enhanced following the administration of GH in rodents (33, 37, 39, 40). Interestingly, the age-dependent increase in plasma cholesterol, as well as the reduced level of BA synthesis that occur in rats, can be completely reversed to the same levels as seen in young animals following GH administration (33), indicating that reduced GH secretion during aging may contribute to the age-dependent rise in serum cholesterol.

We recently found (30) that the pituitary also exerts a strong regulatory function on intestinal cholesterol metabolism in the

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rat. Thus, following hypophysectomy, cholesterol absorption increases by 100%. Although hypophysectomy results in disturbances of hepatic cholesterol metabolism similar to those observed with aging, such as reduced BA synthesis and decreased hepatic expression of LDLRs (40), nothing is presently known about how the relative GH deficiency that occurs with aging relates to intestinal cholesterol absorption.

We hypothesized that an important cause for the hypercholesterolemia present in aged rats could be an increased intestinal absorption of cholesterol, as suggested by previous findings (20, 24, 44). We further reasoned that the relative GH deficiency in aged animals should be an important contributing factor for this, since GH treatment completely reverses the age-induced increase of plasma cholesterol (33). Consequently, treatment of old hypercholesterolemic rats with GH or with ezetimibe (EZE), a specific inhibitor of cholesterol absorption (2, 17), should reverse such an age-induced increase of cholesterol absorption in the intestine and possibly also reduce the hypercholesterolemia in aged rats.

To test this, we directly determined the level of cholesterol absorption in young and old rats as well as the effects of GH and EZE treatment of these animals. Our data confirm that plasma cholesterol is increased in aged rats and that BA synthesis is concomitantly suppressed. These two age-dependent changes are reversed by GH treatment. In contrast, the level of cholesterol absorption is not altered with aging and is not affected by GH treatment. Furthermore, EZE treatment reduced cholesterol absorption to similar extents in both young and old rats. The expression of hepatic LDLRs, scavenger receptors type B class 1 (SR-B1), and the recently discovered receptors type B class 1 (SR-B1), and the recently discovered

E738 AGE-INDUCED HYPERCHOLESTEROLEMIA IN THE RAT

MATERIALS AND METHODS

Animals. A total of 47 male Wistar-Hannover rats (a gerontology rat model from Harlan, Holland) and 24 mo old were used. The animals were 2 and 4 mo old when purchased and were kept in-house until use. The rats were specifically raised for these experiments and were not retired breeder rats. The rats were kept under standardized conditions with free access to water and chow and lights on between 6 AM and 6 PM. The animals were then assigned to six groups: untreated control rats, 10 young and 8 old; GH-treated rats (1.5 mg·kg⁻¹·day⁻¹ bovine GH for 1 wk), 9 young and 9 old; and ezetimibe-treated rats (3 mg·kg⁻¹·day⁻¹ for 12 days), 6 young and 5 old. GH (purchased from Dr. A. F. Parlow, National Hormone and

Assay of serum plant sterols. Sitosterol and campesterol were extracted from 10 μl of serum for each rat in duplicate samples. Samples were derivatized with trimethylsilane reagent (pyridin-hexamethyldisilane-trimethylchlorosilane 3:2:1, vol/vol/vol) prior to gas chromatography-mass spectrometry (GC-MS) analysis (28). D5-campesterol/sitosterol was used as internal standard (25 μl/sample) (28). Plasma plant sterols were corrected for total cholesterol and are expressed as grams of plant sterols per mole cholesterol.

Assay of 7α-hydroxy-4-cholestan-3-one. The concentration of 7α-hydroxy-4-cholestan-3-one (C4) in serum was assayed as described previously (16). In brief, 200 μl of serum was diluted with saline, and 7β-hydroxy-4-cholestan-3-one was added as internal standard. The samples were extracted on C8 Isolute SPE columns (500 mg and 3 ml, International Sorbent Technology, Hengoed, UK) and finally separated by HPLC (HP 1100 series, Hewlett-Packard, Waldbronn, Germany). The wavelength was 241 nm. C4 was corrected for total cholesterol and expressed as milligrams per mole.

Assay of intestinal cholesterol absorption by fecal dual-isotope method. Each rat received a single 0.5-ml corn oil intragastric gavage at 9 PM containing 5 μCi [14C]cholesterol (art. CFA 128; Amersham, Uppsala, Sweden) and 2 μCi [5,6-3H]sitostanol (American Radio-labeled Chemicals art.361) (41). Controls received 0.5 ml of corn oil vehicle with the same procedure. Rats were housed individually in metabolic cages, and stools were collected for 3 days. Twenty-four-hour stools were collected and pooled for each animal and homogenized in PBS. One ml of homogenate was extracted, the ratio of 14C to 3H in feces and in the dosing mixture were determined. The percentage of cholesterol absorbed was calculated for each rat as described (43, 45).

Quantitative real-time PCR. Total RNA was extracted with TRIZol reagent (Invitrogen) according to the manufacturer’s instructions from pooled liver pulverized in liquid nitrogen. One microgram total RNA was transcribed into cDNA by random hexamer priming and Omniscript. Quantitative real-time PCR was performed on triplicate samples of cDNA by use of an ABI Prism 7700 Sequence Detection System (ABI), following the guidelines for SYBR Green assay. Data were corrected for the signal obtained for 18S mRNA in the same cDNA preparation. Primers were designed using Primer Express Software 2.0 (GraphPad Software, San Diego CA, www.graphpad.com).

RESULTS

Cholesterol absorption is not changed with age and not affected by GH treatment. The recent finding that hypophysectomized rats have enhanced intestinal cholesterol absorption (30) led us to hypothesize that the level of intestinal cholesterol absorption is increased in aged rats, since GH secretion is reduced in aged rats (42). To directly study cholesterol absorption, we performed fecal dual-isotope measurements (41) determining the percentage of dietary cholesterol absorbed in the
small intestine in young and old rats as well as in GH-treated animals, as described in MATERIALS AND METHODS. We found that there were no significant changes in the level of cholesterol absorption in aged animals, and that GH-treatment had no effect on cholesterol absorption neither in old nor in younger animals (Table 1). We also measured serum levels of the plant sterol sitosterol and campesterol, reflecting intestinal cholesterol absorption (43) (Table 1). In agreement with the dual-isotope data, the plant sterol levels showed no changes in aged rats or with GH treatment, whereas treatment with EZE, as expected, resulted in drastically and similarly reduced levels of serum plant sterols in both younger and old rats.

Increased serum cholesterol in aged rats can be partly normalized by GH treatment. Analysis of cholesterol lipoprotein profiles by FPLC showed that total cholesterol was 72% higher in the old compared with the younger rats (P < 0.001), in agreement with previous results (33) (Table 2 and Fig. 1A), and importantly, treatment with GH eliminated this age-dependent difference (Fig. 1B). Treatment with EZE did not alter lipoprotein cholesterol levels in young or in old rats (Fig. 1C). Serum triglycerides (TG) were not higher in old compared with young animals, and there were no significant changes induced by either treatment (Table 2).

BA synthesis is reduced with age and restored by GH treatment. We have previously shown that BA synthesis, assessed by measurement of the activity of the rate-limiting enzyme Cyp7a1, is reduced in aged male rats (33). To evaluate the degree of influence of age on BA synthesis in this experiment, we measured serum levels of C4, a BA intermediate that strongly reflects Cyp7a1 activity and BA synthesis (16). Compared with young animals, C4/cholesterol serum levels were clearly reduced in old animals (−59%, P < 0.001; Table 3). Treatment with GH resulted in a strong twofold increase in serum C4/cholesterol in old rats, whereas in young rats this response (+34%) of GH did not reach statistical significance. Comparison of GH-treated young and GH-treated old animals revealed that the C4/cholesterol levels were not different. EZE did not alter C4/cholesterol levels in young or in old rats. Measurement of the mRNA levels for Cyp7a1 did not show any different levels between young and old animals (Table 3), in agreement with previous findings (33). There was, however, a strong increase following GH treatment in young animals, whereas in old animals this stimulation was not statistically significant. EZE treatment did not alter the mRNA levels in young or in old animals.

**DISCUSSION**

A progressive increase in LDL-cholesterol is a well-established part of the complex metabolic changes that occur with normal aging and has potential clinical correlates (1, 5, 6, 12, 19, 26). In humans and in rats, the plasma clearance of LDL is reduced with age (12, 14, 18), presumably due to an altered expression of hepatic LDLRs. The increase in LDL levels seen in older females is generally ascribed to the reduced estrogen response (7, 9), and some studies have shown that shear stress induces LDL absorption by the liver (18).

### Table 1. Intestinal cholesterol absorption in young and old rats and serum plant sterols sitosterol and campesterol adjusted for cholesterol content in serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Young Ctrl (n = 10)</th>
<th>Young + GH (n = 9)</th>
<th>Young + EZE (n = 6)</th>
<th>Old Ctrl (n = 8)</th>
<th>Old + GH (n = 9)</th>
<th>Old + EZE (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol absorption, %</td>
<td>59.5±15.7</td>
<td>53.7±8.2</td>
<td>64.7±8.6</td>
<td>61.3±10.2</td>
<td>7.0±12.9</td>
<td></td>
</tr>
<tr>
<td>Sitosterol/cholesterol, g/mol</td>
<td>12.8±2.1±1b</td>
<td>13.3±2.1±1c,d</td>
<td>12.0±1.8±1c,f</td>
<td>10.7±2.0±1i</td>
<td>7.0±12.9±1d,i</td>
<td></td>
</tr>
<tr>
<td>Campesterol/cholesterol, g/mol</td>
<td>14.3±2.0±1b</td>
<td>14.3±1.8±1c,d</td>
<td>14.3±2.4±1g</td>
<td>13.2±2.3±1k</td>
<td>8.7±19.0±1d,b,k</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. For each measured parameter, identical letters indicate statistical difference at the indicated levels. ab, c, d, e, f, P < 0.001; g, h, i, j, k, P < 0.05. Significances of differences between groups were tested by 1-way ANOVA followed by post hoc comparisons. Data were log transformed for total cholesterol, VLDL-cholesterol, VLDL triglycerides (TG) and for HDL + LDL cholesterol (see MATERIALS AND METHODS).

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**Table 2. Total serum cholesterol and triglycerides and their concentrations in indicated lipoproteins separated by FPLC**

<table>
<thead>
<tr>
<th>Group</th>
<th>Young Ctrl (n = 10)</th>
<th>Young + GH (n = 9)</th>
<th>Young + EZE (n = 6)</th>
<th>Old Ctrl (n = 8)</th>
<th>Old + GH (n = 9)</th>
<th>Old + EZE (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mM</td>
<td>1.39±0.22±1bc</td>
<td>1.69±0.16±1i</td>
<td>1.18±0.14±1c,e</td>
<td>2.40±0.42±1e</td>
<td>2.21±0.61±1e</td>
<td>2.26±0.62±1e</td>
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<tr>
<td>VLDL cholesterol, mM</td>
<td>0.04±0.01±1b</td>
<td>0.07±0.02±1e</td>
<td>0.03±0.02±1d,k</td>
<td>0.12±0.05±1e</td>
<td>0.12±0.07±1d</td>
<td>0.09±0.02±1k</td>
</tr>
<tr>
<td>LDL + HDL cholesterol, mM</td>
<td>1.35±0.22±1b,g</td>
<td>1.62±0.17±1i</td>
<td>1.15±0.16±1d,e</td>
<td>2.31±0.38±1g,k</td>
<td>2.09±0.58±1g</td>
<td>2.17±0.61±1g,e,s</td>
</tr>
<tr>
<td>Total TG, mM</td>
<td>0.89±0.39</td>
<td>1.33±0.37</td>
<td>1.25±0.47</td>
<td>1.35±0.89</td>
<td>1.12±0.82</td>
<td>1.64±0.40</td>
</tr>
<tr>
<td>VLDL TG, mM</td>
<td>0.34±0.20</td>
<td>0.59±0.18</td>
<td>0.60±0.27</td>
<td>0.55±0.40</td>
<td>0.48±0.21</td>
<td>0.72±0.20</td>
</tr>
<tr>
<td>LDL + HDL TG, mM</td>
<td>0.54±0.20</td>
<td>0.74±0.21</td>
<td>0.65±0.20</td>
<td>0.80±0.49</td>
<td>0.64±0.20</td>
<td>0.92±0.20</td>
</tr>
</tbody>
</table>

Values are means ± SD. For each parameter, identical letters indicate statistical difference at the indicated levels. ab, c, d, e, f, P < 0.001; g, h, i, j, k, P < 0.05. Significances of differences between groups were tested by 1-way ANOVA followed by post hoc comparisons. Data were log transformed for total cholesterol, VLDL-cholesterol, VLDL triglycerides (TG) and for HDL + LDL cholesterol (see MATERIALS AND METHODS).
followed by posthoc comparisons. Data were log transformed for C4/cholesterol, (see MATERIALS AND METHODS).

A difference at the indicated levels: a,b,c,d,e,f -hydroxylase; PCSK9, proprotein convertase subtilisin/kexin type 9 serine protease. For each measured parameter, identical letters indicate statistical

Serum C4 levels and analysis of hepatic mRNA expression of young and old rats corrected for 18S

Exerts a stimulatory effect on hepatic LDLR expression in most species (3, 23, 29). The facts that the pituitary secretion of GH is essential for this estrogen-induced stimulation and that the GH secretion pattern is influenced by estrogen, together with the known reduction in GH secretion in both sexes during normal aging (9, 42), have led to the hypothesis that a relative deficiency in GH may, at least in part, explain the age-related increase in plasma LDL that occurs in both sexes (38). There is also evidence of a reduced synthesis of BAs with increasing age in the rat (33), and possibly also in humans (10). In rodents, but apparently not in humans (27, 32), GH is important for the basal activity of Cyp7a1 and thus for the regulation of cholesterol breakdown to BAs. In line with this thinking, the administration of GH to old rats results in a stimulation of Cyp7a1 and a normalization of the plasma lipoprotein pattern (33). Stimulation of hepatic LDLRs and an increased clearance of plasma LDL by GH has also been demonstrated in normal adult humans (27, 39).

In recent studies, we have found evidence of an increased intestinal absorption of cholesterol in hypophysectomized rats (30). This finding appears to be of major importance in explaining why hypophysectomized rats are extremely sensitive to cholesterol/fat feeding. The capacity to handle dietary cholesterol/fat may also be reduced with increasing age and contribute to the increased levels of plasma LDL (14). However, the literature on how aging influences cholesterol and fat absorption, in both animals and humans, is limited, and to some extent, contradictory (13, 21, 44). In the present work, we tested the hypothesis that cholesterol absorption, which is under control by the pituitary (30), might be one important explanation for the increased plasma cholesterol levels present in old rats.

Somewhat to our surprise, we could not find any difference between young and old rats with respect to intestinal cholesterol absorption, either when determined by the fecal dual-isotope technique or by assaying the levels of serum plant sterols (31). By blocking the activity of the cholesterol transporter NPC1L1 through treatment with the specific cholesterol absorption inhibitor EZE (17), we could, as then expected, reduce cholesterol absorption equally well in young and old animals, as evaluated from the levels of serum plant sterols. It should be noted that in this study EZE did not reduce the elevated plasma cholesterol present in old rats. This finding is important, as it further supports the notion that a change in cholesterol absorption is not of major importance for the age-dependent increase in plasma cholesterol in the rat. The lack of effect of EZE on plasma cholesterol in normal rats is in line with results of Repa et al. (36) using a similar compound.

This study clearly showed that other mechanisms than altered cholesterol absorption explain why serum cholesterol increases in old rats. The age-dependent increase in serum cholesterol in old Wistar-Hannover rats was reduced following GH treatment, a response present only in old animals and not in young rats, which is in close agreement with previous results on Sprague-Dawley rats (33). Cyp7a1 was reduced in old animals also in this strain of rats and could be restored to normal (young) levels after substitution with GH. Also in agreement with previous results where LDLR mRNA and protein expression were assayed (33), LDLR mRNA levels were unaltered in aged rats, as was the gene expression of

<table>
<thead>
<tr>
<th>Table 3. Serum C4 levels and analysis of hepatic mRNA expression of young and old rats corrected for 18S</th>
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<tbody>
<tr>
<td>Group</td>
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<tr>
<td>-------</td>
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<tr>
<td>C4cholesterol, mg/mol</td>
</tr>
<tr>
<td>LDLR/18S mRNA</td>
</tr>
<tr>
<td>SR-BI/18S mRNA</td>
</tr>
<tr>
<td>PCSK9/18S mRNA</td>
</tr>
<tr>
<td>Cyp7a1/18S mRNA</td>
</tr>
</tbody>
</table>

Values are means ± SD. LDLR, LDL receptor; C4, 7α-hydroxy-4-cholesten-3-one; SR-BI, scavenger receptors type B class 1; Cyp7a, cholesterol 7α-hydroxylase; PCSK9, proprotein convertase subtilisin/kexin type 9 serine protease. For each measured parameter, identical letters indicate statistical difference at the indicated levels: a,b,c,d,e,f,p < 0.001; g,h,p < 0.01; k,m,p < 0.05. The significance of differences between groups was tested by 1-way ANOVA followed by posthoc comparisons. Data were log transformed for C4/cholesterol, (see MATERIALS AND METHODS).
SR-BI and PCSK9. The finding that GH infusion stimulated the gene expression of PCSK9 suggests that, in parallel to the induction of hepatic LDL receptors and LDLR mRNA following the infusion of GH to rats (33), there is a counterregulatory action by PCSK9 that will dampen the expression of LDLRs. Such a response has previously been shown to occur following treatment with statins (35).

Whereas GH is undoubtedly important in the regulation of plasma and hepatic lipid metabolism in aging intact rats, this hormone was not of major importance for the level of intestinal cholesterol absorption. It should be pointed out that, similar to our previous studies (33), our experiments were limited to male rats, and we cannot exclude the presence of sex-related differences in the development of hypercholesterolemia with aging.

In conclusion, reduced bile acid synthesis likely contributes to the age-related dyslipidemia in the rat. Bile acid synthesis can be restored (to “young” levels) by GH administration, indicating a possible role of a relative GH deficiency in the old rats. Although hypophysectomized rats display increased cholesterol absorption (30), this is clearly not a feature of the aging rat, and GH treatment was without effect on cholesterol absorption in young or in old rats.

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