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

Cecilia Gálman,1,*, Manuela Matasconi,1,*, Lena Persson,1 Paolo Parini,2 Bo Angelin,1 and Mats Rudling1

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 atherosclerosis 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...
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 LDLR-degrading serine protease PCSK9 was unaltered with age. Hepatic LDLR and PCSK9 mRNA levels were, however, induced by GH independently of age.

MATERIALS AND METHODS

Animals. A total of 47 male Wistar-Hannover rats (a gerontology rat model from Harlan, Holland) 6 and 18 mo old were used. The animals were 2 and 14 mo old when purchased and were kept in-house until use. The rats were specifically raised for these experiments and thus 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 Peptide Program NHPP, Harbor-UCLA Medical Center, Torrance, CA) was infused subcutaneously by surgically implanted miniosmotic pumps (model 2ML1; Alzet, Palo Alto, CA) as previously described (33). Ezetimibe (Ezetrol; MSD-SP, Hoddesdon, UK) was mixed with the food. The rats were killed by decapitation during isoflurane anesthesia, and trunk blood was collected. The use of trunk blood is appropriate because the level of the compounds analyzed are similar in arterial and venous blood. Serum was obtained and stored at −80°C. Livers, proximal small intestines, and distal ileums were frozen in liquid nitrogen and kept at −80°C. Liver and body weights were recorded. The study was approved by the Karolinska Institute Institutional Animal Care and Use Committee.

Assay of serum lipids. Serum lipoproteins were size-fractionated using 10 µl of serum from each individual animal by a fast performance liquid chromatography system (FPLC) as previously described (34). In the rat, LDL and HDL overlap strongly, and therefore attempts to calculate absolute levels of these fractions will be erroneous.

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-hexamethyldisililane-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-cholesten-3-one. The concentration of 7α-hydroxy-4-cholesten-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-cholesten-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 [5,6,3HJSitostanol (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 wastranscribed 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 (ABI); sequences: LDLR (700 nM): 5′-GGGTTCATACGGTTTC- GT-3′ and 5′-TGTTATCTCGCTGCGGC-TC3′; SR-BI (200 nM): 5′-GTTTCCTAGACATCCACCCGGT-3′ and 5′-TGTTACAGACTCAGCTGATCTTCA-3′; PCSK9 (200 nM) 5′-GCACCTGGAGACCAACAA- CAGG-3′ and 5′-TGGCTCATGATACCTTCTTCTC-3′; 18S (100 nM): 5′-CCTGGGCTTAAATTTGACTCA-3′ and 5′-AGCTATCAATCTGTCAATCCGTCC-3′.

Statistics. The significance of differences between groups in Tables 1–3 was tested by one-way ANOVA followed by post hoc comparisons according to Tukey, using GraphPad Prism version 4.03, (GraphPad Software, San Diego CA, www.graphpad.com).

Data were log transformed when there was a clear positive correlation (P < 0.05) between group means and group SD; this was the case for C4/cholesterol, campesterol/cholesterol, total cholesterol, VLDL-cholesterol, VLDL triglycerides, and HDL + LDL cholesterol.

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
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±1.2</td>
<td></td>
</tr>
<tr>
<td>Sitosterol/cholesterol, g/mol</td>
<td>12.8±2.1</td>
<td>13.3±2.1</td>
<td>12.0±1.8</td>
<td>10.7±2.0</td>
<td>7.0±1.2</td>
<td></td>
</tr>
<tr>
<td>Campesterol/cholesterol, g/mol</td>
<td>14.3±2.0</td>
<td>14.3±1.8</td>
<td>14.3±2.4</td>
<td>13.2±2.3</td>
<td>8.7±1.9</td>
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</tbody>
</table>

Values are means ± SD. For each measured parameter, identical letters indicate statistical difference at the indicated levels. **P < 0.001; *P < 0.01; ^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).

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.

**Aging does not alter LDLR, SR-BI, or PCSK9 mRNA levels.**

To establish whether changes in the expression of hepatic genes other than Cyp7a1 could explain serum lipid changes in old rats, we quantified mRNA levels of the lipoprotein receptors LDLR and SR-BI and of the LDLR-degrading protease PCSK9 (Table 3). The gene expression of both lipoprotein receptors as well as of PCSK9 was, however, unaltered in aged rats. GH treatment increased LDLR and PCSK9 mRNA levels in both young and old rats. Treatment with EZE had no effect on the gene expression of the lipoprotein receptors or PCSK9. Thus, the age-related increases in serum cholesterol and its reduction by GH were only associated with reciprocal changes in hepatic Cyp7a1 enzymatic activity as evaluated from its plasma marker C4.

**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 levels following menopause, since it is known that estrogen strongly reflects Cyp7a1 activity and BA synthesis (16). Cyp7a1 activity is associated with age and is partly explained by the fecal dual-isotope method as described in MATERIALS AND METHODS. For each measured parameter, identical letters indicate statistical difference at the indicated levels. **P < 0.001; *P < 0.01; ^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).
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 cholesterol 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 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).

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Table 3. Serum C4 levels and analysis of hepatic mRNA expression of young and old rats corrected for 18S

<table>
<thead>
<tr>
<th>Group</th>
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<th>Old + EZE (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4/cholesterol, mg/mol</td>
<td>23.9±10.1±g</td>
<td>32.0±9.6±h</td>
<td>23.9±1.9±g</td>
<td>11.0±1.7±b,d,e,f</td>
<td>27.3±12.2±d</td>
<td>11.5±1.7±f,g,h</td>
</tr>
<tr>
<td>LDLR/18S mRNA</td>
<td>0.78±0.21±h</td>
<td>1.29±0.24±e</td>
<td>0.93±0.26±h</td>
<td>0.86±0.20±g</td>
<td>1.32±0.16±b,h,m</td>
<td>1.01±0.14±m</td>
</tr>
<tr>
<td>SR-BI/18S mRNA</td>
<td>1.12±0.19</td>
<td>1.13±0.21</td>
<td>0.88±0.31</td>
<td>1.03±0.19</td>
<td>0.97±0.17</td>
<td>0.85±0.24</td>
</tr>
<tr>
<td>PCSK9/18S mRNA</td>
<td>0.60±0.15±b,g</td>
<td>1.48±0.17±c,h</td>
<td>0.92±0.23±b,i</td>
<td>0.71±0.31±c,d</td>
<td>1.41±0.25±b,d,l</td>
<td>1.16±0.21±f</td>
</tr>
<tr>
<td>Cyp7a1/18S mRNA</td>
<td>0.58±0.28±g</td>
<td>1.85±0.95±b,c,h</td>
<td>0.63±0.40±g</td>
<td>0.48±0.2±h</td>
<td>1.25±0.61±k,m</td>
<td>0.37±0.11±c,m</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; Cyp7a1, 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 < 0.001; g,h,i < 0.01; k,m < 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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REFERENCES


