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Modified HMG-CoA Reductase and LDLr Regulation is Deeply Involved in Age-Related Hypercholesterolemia

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Abstract During the ageing process in rats hypercholesterolemia occurs in concert with full activation, lowered degradation rate and an unchanged level of the rate limiting cholesterol biosynthesis enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR). The molecular bases of the HMG-CoAR unchanged level and lowered degradation rate in aged rats is not clear. In fact no data are available during ageing, on transcription and degradation of HMG-CoAR, so well defined in adult animal. So, aim of this work was to measure mRNA levels of the enzyme and the level of the proteins of the regulatory complex responsible of the cholesterol metabolism. To complete the picture, the level of sterol regulatory element binding proteins (SREBPs), SREBP cleavage activating protein, and insulin-induced gene has been measured. The levels of other related proteins, whose transcription is SREBP dependent, that is low density lipoprotein receptor (LDLr) and Caveolin 1, have been also measured. The age-related reduced Insigs levels, joined to a reduced insulin sensitivity, could explain the decreased degradation rate of the HMG-CoAR and the increased active SREBP-2. The SREBP-2 in particular seems to be committed in multiple way to gene transcription. The obtained data represent a good contribution to explain the age-related hypercholesterolemia. J. Cell. Biochem. 98: 1044–1053, 2006.

Key words: ageing; cholesterol; HMG-CoA reductase; Insig; LDLr; SCAP; SREBPs; rat liver

Ageing is characterized by several metabolic changes responsible for the decline of some functions and the appearance of age related diseases. The enhanced content of cholesterol in the blood is retained to be one of the most dangerous features since this compound is considered responsible for atherosclerosis and other heart diseases.

Cholesterol is an isoprenoid compound that plays an important role in modulating fluidity and phase transitions in the plasma membrane of animal cells [Devaux, 1993]. It participates in the formation of plasma membrane rafts and caveolae, both sites where signaling molecules concentrate [Simons and Ikonen, 1997; Anderson, 1998]. However, an excessive amount of cholesterol in the blood can destroy membrane function or result in atherosclerotic damage [Small and Shipley, 1974]. Liver is the principal site for cholesterol homeostasis maintenance [Dietschy et al., 1993], carried out in many mechanisms, such as the biosynthesis, via 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR, EC 1.1.1.34) activity, the uptake through lipoprotein receptors, the release in the blood by lipoproteins, the storage by esterification, the degradation and the conversion into bile acids [Weber et al., 2004].

HMG-CoAR is an integral membrane protein of the endoplasmic reticulum (ER). It constitutes the key enzyme, catalyzing the rate-limiting

Abbreviations used: AMPK, AMP-activated kinase; Chol, cholesterol; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; Insig, INSulin induced gene; HDL, high density lipoprotein; IRS, insulin receptor substrate; LDLr, low density lipoprotein receptor; PP2A, protein phosphatase 2A; SCAP, SREBP cleavage activation protein; SREBPs, sterol regulatory element binding proteins.

Valentina Pallottini and Chiara Martini have contributed equally to this manuscript.

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reaction of cholesterol and other isoprenoid biosynthesis (i.e., ubiquinon, squalene, dolichol, and prenylated proteins) and it is regulated at multiple levels [Geelen et al., 1986; Goldstein and Brown, 1990]. Long-term regulation is guaranteed by the control of synthesis and degradation of the enzyme. Short-term regulation is mainly assured by phosphorylation and dephosphorylation processes. Specific kinases and phosphatases, essentially AMP-activated kinase (AMPK) and protein phosphatase 2A (PP2A), are responsible for the active/inactive enzyme interconversion [Ching et al., 1996; Gaussin et al., 1997].

Long-term regulation is assured both by final product feedback inhibition and by hormones that control HMG-CoAR transcription and degradation. HMG-CoAR gene transcription is guaranteed by sterol regulatory element binding proteins (SREBPs) that belong to the large family of basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors. Three members of the SREBP family, SREBP-1a, -1c, and -2, have been identified [Hua et al., 1993; Yokoyama et al., 1993]. They are synthesized as inactive precursors in ER. Each nascent SREBP protein has a molecular size of approximately 125 kDa, and consists of about 1,150 aminoacids organized in three functional domains. The first domain, the NH_2-terminal domain of SREBP, protrudes into the cytosol, and contains the bHLH-Zip and acidic domain that has to bind a transcription coactivator to function [Sato et al., 1994]. The central portion of the molecule consists of two membrane-spanning domains that anchor SREBP to the ER and nuclear envelope. The COOH terminal segment of 590 aminoacids extends into the cytosol and serves as the regulatory domain for the transformation into its mature, transcriptionally active form, also known as nuclear SREBP (nSREBP). The 68-kDa nSREBP binds to the sterol regulatory element (SRE) which is present in about 30 of the genes involved in the lipid metabolism, such as HMG-CoAR, low density lipoprotein receptor (LDLr) genes [Horton et al., 2002], and Caveolin 1 gene [Bist et al., 1997; Cao et al., 2005]. This then activates the transcription.

SREBPs attain biological activity after being transferred to the membrane Golgi complex, where the NH_2-end is released from the membrane following the enzymes' cleaving at the luminal loop. SREBPs are confined to the ER membranes until they are transported to the Golgi complex by an escort protein required for their cleavage and activation, the SREBP cleavage activation protein (SCAP). SCAP has 1,276 aminoacids and consists of two domains: a membrane-spanning NH_2-terminal domain and a COOH terminal domain, which extends in the cytosol and mediates SCAP/SREBP interaction [Sakai et al., 1997].

Two ER membrane proteins play a central role in the feedback control of lipid synthesis in animal cells: INSulin induced Gene -1 and -2 (Insig-1 and Insig-2). The transcription of these proteins, modulated by insulin, regulates lipid content controlling both HMG-CoAR transcription and degradation in a sterol dependent way. Sterol-stimulated binding of Insigs to SCAP leads to the retention of the SCAP/SREBP complex in ER, preventing the proteolytic generation of the transcriptionally active nSREBPs [Horton et al., 2002]. Furthermore, sterol-stimulated binding of Insigs to HMG-CoAR leads to its ubiquitination and proteasomal degradation [Sever et al., 2003a,b].

HMG-CoAR is physiologically present in the cell in an unphosphorylated active form and a phosphorylated inactive form. The ratio between active and total forms indicates the activation state of HMG-CoAR. In 24-month-old ad libitum (AL) fed rats, it has been shown that the hepatic HMG-CoAR is fully activated, cholesterol synthesis increased, with cholesterol content unchanged in the liver but increased in the blood [Marino et al., 1998, 2002]. Moreover, the protein level of HMG-CoAR is not modified but the degradation rate is slowed and not influenced by mevalonate and 25-hydroxycholesterol, two well-known modulators of its degradation [Pallottini et al., 2003, 2004].

The pattern of the factors involved in the regulation of HMG-CoAR transcription and degradation has been well defined in the adult animal but no data are available in old one.

The aim of this work was to draw a picture of the protein complex responsible for the regulation of the cholesterol metabolism during the ageing process. To clarify the molecular bases of the HMG-CoAR unchanged level and lowered degradation rate, its mRNA level, along with Insigs, SREBPs, and SCAP protein levels have been measured and the insulin responsiveness of the aged model checked. The levels of other cholesterol metabolism related proteins whose

transcription is SREBP dependent, as well as LDLr and Caveolin 1, have been also measured.

MATERIALS AND METHODS

Materials

All chemicals used were obtained from Sigma Aldrich (Milano, Italy) and were of the highest quality available.

Animals

Random groups of 3- and 24-month-old male Sprague–Dawley rats were maintained on standard laboratory food (Harlan autocavable Teklad diet, Harlan Italy S.r.l., containing: 12.0% water, 18.4% crude protein, 5.5% crude fiber, 5.6% crude ash). Rats had free access to water. Before the sacrifice, the animals were anaesthetized with intraperitoneal injection of pentobarbital (50 mg/kg body weight) and subjected to liver excision. Livers were immediately frozen into liquid nitrogen and stored at −80°C.

The Official Italian Regulation N. 116/92 for the care and use of laboratory animals was followed.

Plasma Cholesterol Content Assay

Blood was collected on EDTA (0.4 mg/ml blood). Plasma was separated by centrifugation. The analysis of total and lipoprotein linked plasma cholesterol (Chol) was performed using the diagnostics kits for Total-Chol, high density lipoprotein-chol (HDL-Chol), and LDL-Chol (Sigma Chemical Co.).

Western Blot Analysis

Insig-1, Insig-2, SREBP-1 (C-terminus), SREBP-2 (C-terminus), and SCAP. The Western blot analysis of Insig-1, Insig-2, SREBP-1 (C-terminus), SREBP-2 (C-terminus), and SCAP were performed on microsomes prepared as described Bruscalupi et al. [1985], then solubilized in 0.125 M Tris-HCl (pH 7.8) containing 10% SDS, 1 mM phenylmethylsulphonyl fluoride (sample buffer), and boiled for 2 min. Protein concentration was determined by the method by Lowry et al. [1951]. Twenty micrograms of protein from solubilized microsomes were resolved by 12% (for Insigs) and 7% (for SREBPs and SCAP) SDS–PAGE at 100 V for 60 min. The proteins were subsequently transferred electrophoretically onto nitrocellulose for 80 min at 100 V. The nitrocellulose was treated with 3% bovine serum albumin in 138 mM NaCl, 27 mM KCl, 25 mM Tris-HCl, 0.05% Tween-20 (pH 6.8) (block buffer), and probed at 4°C overnight with Insig-1 and -2 antibody (Southwestern Medical Center, University of Texas, Dallas, TX, USA), SREBP-1 and -2, and SCAP antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For protein detection, secondary anti-rabbit antibodies for SREBP-1, anti-mouse for Insig-1 and -2, anti-goat for SREBP-2 and SCAP (Santa Cruz Biotechnology) were used. Bound antibodies were visualized using enhanced chemoluminescence detection (ECL, Amersham-Pharmacia, Little Chalfont, UK).

Insulin Receptor Substrate

Western blot analysis of insulin receptor substrate (IRS) was performed on total liver lysate obtained as follows: one hundred milligrams of liver tissue were solubilized by sonication in sample buffer in presence of 50 mM NaF to avoid dephosphorylation, and boiled for 2 min. Protein concentration was determined using the method by Lowry et al. [1951]. Twenty micrograms of proteins from solubilized tissue were resolved by 7% SDS–PAGE at 100 V for 60 min, and subsequently transferred electrophoretically as described above by probing at 4°C overnight with anti-P-IRS 1/2 (Santa Cruz Biotechnology) and then stripped and probed with anti-actin (Santa Cruz Biotechnology). For protein detection secondary anti-rabbit antibodies for IRS, and secondary anti-mouse for actin, were used. Bound antibodies were visualized as described above.

Nuclear SREBP-1 and Nuclear SREBP-2

The Western blot analyses of nSREBP-1, nSREBP-2 were performed on nuclei prepared as follows: five hundred milligrams of liver were homogenized in sucrose 0.25 M (10% w/v), and then centrifuged 10 min at 1,000*g*. The pellet was washed three times at the same speed in sucrose solution to obtain nuclear fraction. Nuclei were solubilized in sample buffer, and boiled for 2 min. Twenty micrograms of protein from solubilized nuclei were resolved by 10% SDS–PAGE at 100 V for 60 min. The proteins were subsequently transferred electrophoretically as described above. The nitrocellulose was treated with block buffer, probed at 4°C

overnight with SREBP-1 (N-terminus) and -2 (N-terminus) (Santa Cruz Biotechnology).

For protein detection, secondary anti-rabbit antibodies for SREBP-1, anti-mouse for SREBP-2 were used. Bound antibodies were visualized as described above.

LDLr and Caveolin 1

The Western blot analysis of LDLr was performed on total membrane obtained as follows: five hundred milligrams liver tissue were homogenized in 0.01 M Tris-HCl, 0.001 M $CaCl_2$, 0.15 M NaCl, 0.001 M PMSF pH 7.5. Homogenate was centrifuged for 10 min at 500*g*, the supernatant centrifuged again 10 min at 500*g*. The supernatant was centrifuged at 100.000*g* for 30 min and the pellet was resuspended and centrifuged again at 100.000*g* for 20 min. The pellet was resuspended, aliquots were transferred in 1.5 ml Eppendorf tubes and centrifuged for 3 min at 14.000 rpm. The pellets were resuspended in sample buffer for the protein determination by Lowry et al. [1951] method and for 7% SDS–PAGE. The proteins separation and blot were performed as described above. LDLr and Caveolin 1 detection were made probing the nitrocellulose with polyclonal antibodies (Santa Cruz Biotechnology). For LDLr detection we used secondary anti-goat antibodies (Santa Cruz Biotechnology), and for Caveolin 1 detection anti-rabbit (Santa Cruz Biotechnology). Bound antibodies were visualized as described above.

Normalization of Protein Measured

The normalization of the analyzed protein content has been performed only where was possible to use anti-actin antibody, (i.e., in the total lysate).

mRNA Levels

HMG-CoAR and LDLr gene expression was performed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from 50 mg of rat hepatic tissue was isolated with TRI-Reagent (Mol. Res. Centre Inc. Cincinnati, OH), following the manufacturer's instruction. Briefly, the tissue was homogenized in 0.25 ml of cold 0.9% NaCl; then, 0.75 ml of TRI-Reagent and 0.2 ml of chloroform were added to the homogenate. The samples were vigorously shaken and centrifuged and the RNA present in the aqueous phase was precipitated with 0.5 ml of isopropanol. The RNA pellet was washed once with 1 ml of 75% ethanol, dried, resuspended in sterile water and quantified by UV absorbance. Two micrograms of total RNA were used for the reverse transcription reaction performed in 20 μl of final volume at 41°C for 60 min, using 30 pmol of antisense primer (Table I) for analyses of the HMG-CoAR, the LDL-R and the β-actin gene. Rat β-actin gene was utilized as an internal control and it was chosen as a reference gene because it is a housekeeping gene. Real-time PCRs were performed in 25 μl of final volume containing 2 μl of cDNA, master mix with SYBR Green (iQ SYBR Green Supermix Bio-Rad, Milan, Italy) and sense and antisense primers for each tested gene. Each gene was analyzed in separated tubes.

Real-time PCR was carried out in iCycler Thermal Cycler System apparatus (Bio-Rad) using the following parameters: one cycle of 95°C for 1 min and 30 s, followed by 45 cycles at 94°C for 10 s, 55°C for 10 s, and 72°C for 30 s and a further melting curve step at 55–95°C with a heating rate of 0.5°C per cycle for 80 cycles. The

TABLE I. Sequences of Amplification Primers

Gene		Primer
HMG-CoAR	Sense	5′-GCTTGAGCATCCTGACATAC-3′
	Antisense	5′-GAACCATAGTTCCCACGTCT-3′
LDL-R	Sense	5′-CGAGTTCCAGTGTAGAGACG-3′
	Antisense	5′-GGGAGCAGTCTAGTTCATCC-3′
β-actin	Sense	5′-GCCTCTGGTCGTACCACTGGC-3′
	Antisense	5′-AGGGAGGAAGAGGATGCGGCA-3′

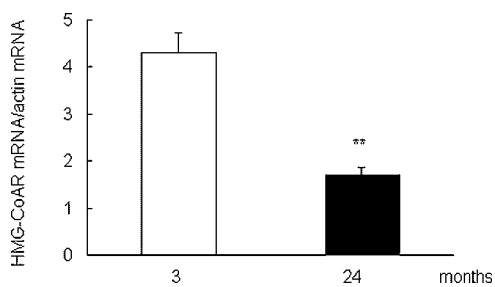

Fig. 1. HMG-CoAR mRNA amount in adult and old rat liver. The Figure shows HMG-CoAR mRNA level analyzed by real-time RT-PCR. Hepatic total RNA was reverse-transcripted and subsequently amplified by real-time PCR by using master mix with SYBR Green and sense and antisense primers for the tested gene (Table I). For details see the text. The bars represent the ratio between the number of HMG-CoAR mRNA molecules and the number actin mRNA molecules in old (24 months) rats and adult (3 months) rats. The analysis was performed on four different experiments. $^{**}P < 0.001$ as from a Student's *t* test with the respect to adult (3 months).

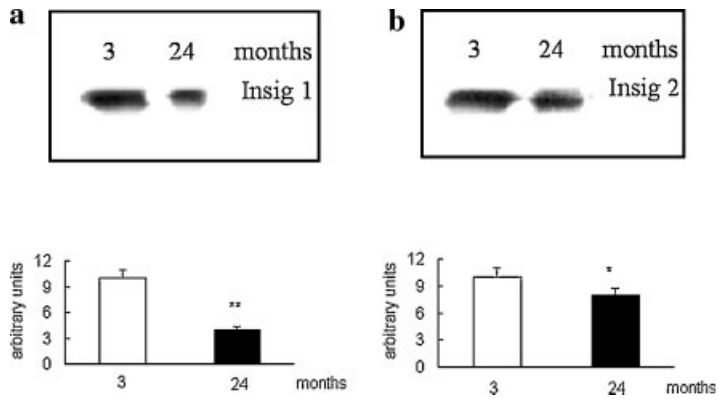

Fig. 2. Insig-1 and Insig-2 protein levels in adult and old rat liver. Western blots showing Insig-1 (**panel a**) and Insig-2 (**panel b**) levels in adult (3 months) and old (24 months) rat liver microsomes. Proteins from 20 μg solubilized liver microsomes were resolved by SDS–PAGE, and subsequently transferred electrophoretically onto nitrocellulose and probed with anti-Insig-1 and -Insig-2 polyclonal antibodies. For details see the text. Panels show a typical Western blotting and the densitometric analysis obtained from four different experiments. $^{*}P < 0.05$ as from a Student's t test with the respect to adult (3 months). $^{**}P < 0.001$ as from a Student's t test with the respect to adult (3 months).

PCR products were quantified by external calibration curves, one for each tested gene, obtained with serial dilutions of known copy number of molecules (10^2–10^7 molecules). All expression data were normalized by dividing the amount of target by the amount of β-actin used as internal control for each sample. The specificity of the PCR product of each tested gene was confirmed by gel electrophoresis.

Statistical Analysis

Data are expressed as means and standard deviation, and intergroup differences were analyzed using the Student's t test.

RESULTS

In order to study the mechanism underlying age-related HMG-CoAR behavior, the long-term regulation has been studied and the levels of the known factors involved have been measured.

First of all, to relate the unchanged HMG-CoAR protein level with the lower degradation rate of the enzyme observed in the liver of 24 months old rat [Pallottini et al., 2003, 2004] its mRNA level has been measured. A lower amount of mRNA is detectable in 24-month-old rat liver with respect to 3-month-old ones (Fig. 1). This datum indicates either a lower transcription or a lower mRNA stability which is in accord with the unchanged level of the reductase observed along with the decreased degradation rate.

Because of Insigs involvement, both in the enzyme gene transcription and in the enzyme protein degradation, their protein level in adult and aged rat liver, has been measured. As shown in Figure 2a, a large decrease of Insig-1 and a less remarkable reduction of Insig-2 levels (Fig. 2b) are detectable in aged rats. Since Insig-1 mRNA is reported to be induced directly or indirectly by insulin [Yang et al., 2002; Kast-Woelbern et al., 2004] the sensitivity to this

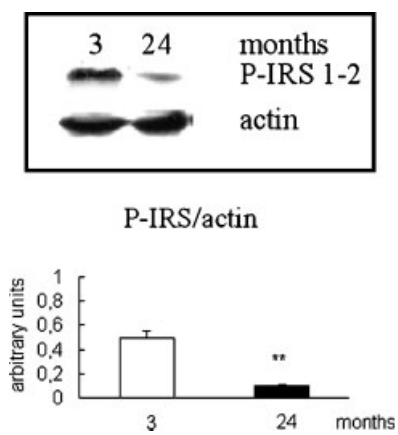

Fig. 3. P-IRS 1-2 in adult and old rat liver. The figure represents on the top a typical Western blotting, on the bottom the ratio between the densitometric analysis of the P-IRS 1-2 levels and actin obtained from four different experiments. The experiments were performed in adult (3 months) and old (24 months) rat liver total lysates. Proteins from 20 μg total liver lysate were resolved by SDS–PAGE, subsequently transferred electrophoretically onto nitrocellulose and then probed with anti-P-IRS 1-2 antibody then stripped and probed with anti-actin antibody. For details see the text. $^{**}P < 0.001$ as from a Student's t test with the respect to adult (3 months).

hormone in old and adult rat liver has been checked by measuring the IRS phosphorylation as a signal of insulin receptor activation. The strong reduction of IRS 1-2 phosphorylation detectable in old rat liver (Fig. 3) confirms a lower sensitivity to insulin in old rat liver, as elsewhere described [Fulop et al., 2003; Moller et al., 2003; Masoro, 2005].

Insig-1 and Insig-2 are involved in HMG-CoAR transcriptional regulation because of their ability to bind the SCAP/SREBP complex, thus avoiding the translocation of such a complex to the Golgi apparatus and the formation of mature SREBP. Consequently, the levels of nSREBPs in nuclei and the levels of their precursors, SREBPs, in microsomes have been measured. Figure 4 shows an increase of nSREBP-2 levels, not significant changes in nSREBP-1 levels and a decrease in SREBP-2 and -1 precursor form in ER. With relation to SCAP, an enhanced level in ER was detected (Fig. 5).

The increase of nSREBP-2 suggests a putative stimulation of nSREBP-2 dependent genes. Besides the already mentioned HMG-CoAR mRNA, the LDLr mRNA levels have been analyzed. The results show that while HMG-CoAR mRNA level resulted lower in an aged liver with the respect to an adult one, on the contrary LDLr mRNA level increases in 24-month-old animals (Fig. 6a). Amongst the SREBPs target genes, the LDLr is particularly interesting because of its involvement with cholesterol metabolism [Horton et al., 2002]. The analysis of the protein level in aged rats shows an increase of LDLr in total liver lysate in accordance with its mRNA level (Fig. 6b), and a decrease of LDLr on liver membranes (Fig. 6c). A low Caveolin 1 content is also observable (Fig. 7) perfectly consistent with mRNA content

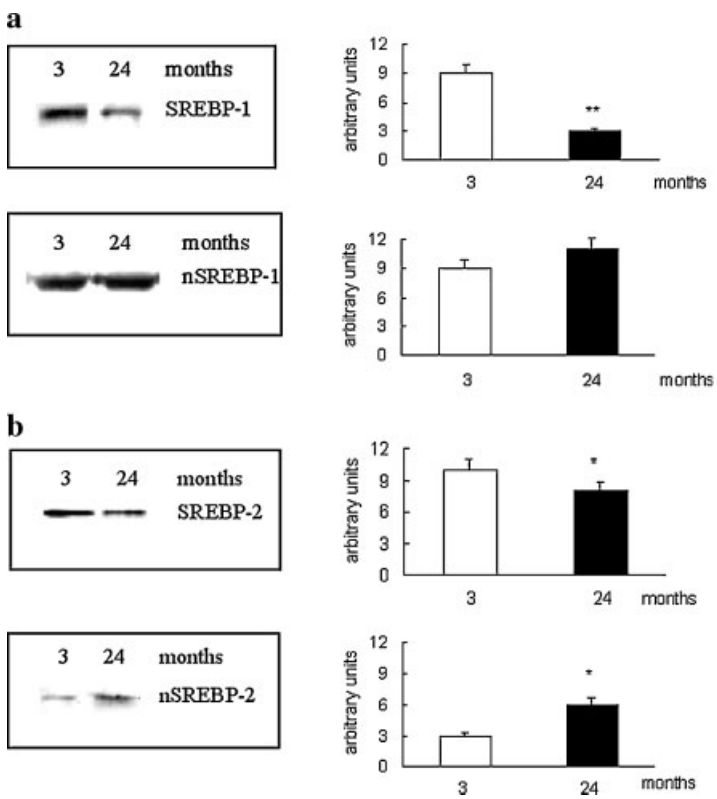

Fig. 4. SREBPs protein levels in adult and old rat liver. **Panel a** illustrate Western blots showing on the top microsomal SREBP-1, on the bottom nuclear SREBP-1 with the densitometric analysis obtained from four different experiments. **Panel b** illustrate Western blots showing on the top microsomal SREBP-2, on the bottom nuclear SREBP-2 with the densitometric analysis obtained from four different experiments. The experiments were performed in adult (3 months) and old (24 months) rat liver. Microsomes and nuclei were obtained as described in the Material and Methods section. Proteins from 20 μg microsomes or solubilized nuclei were resolved by SDS–PAGE and subsequently transferred electrophoretically onto nitrocellulose then probed with primary antibodies as described in the text. $^{*}P < 0.05$ as from a Student's *t* test whit the respect to adult (3 months). $^{**}P < 0.001$ as from a Student's *t* test with the respect to adult (3 months).

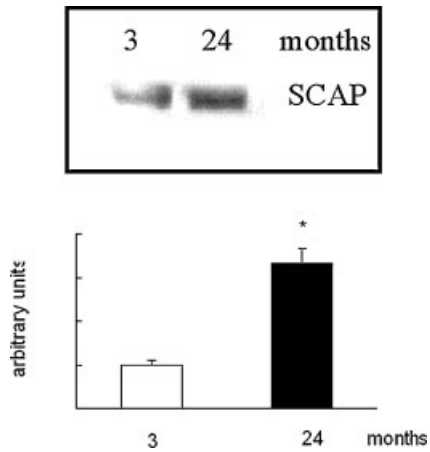

Fig. 5. SCAP protein level in adult and old rat liver. The figure represents on the top a typical Western blotting, on the bottom the densitometric analysis of the SCAP levels obtained from four different experiments. The experiments were performed in adult (3 months) and old (24 months) rat liver microsomes. Proteins from 20 μg microsomes were resolved by SDS–PAGE, subsequently transferred electrophoretically onto nitrocellulose and then probed with anti-SCAP antibody. For details see the text. $^*P < 0.05$ as from a Student's t test with the respect to adult (3 months).

already reported by Mulas et al. [2005]. Caveolin 1 is another product of a SREBP-dependent gene transcription whose presence on plasma membrane is strongly related to membrane LDLr localization [Ness et al., 2003]. The reduced-LDLr presence on membrane could be connected to the already reported hypercholesterolemia [Marino et al., 2002]. The data shown in Table II confirm that cholesterol, both total and that linked to lipoproteins, strongly increases in aged rat plasma.

DISCUSSION

During ageing, hypercholesterolemia is usually an observable feature. Alteration of cellular cholesterol homeostasis appears to play a major role in atherosclerosis and cardiac disease.

Since cholesterol homeostasis results from the interaction of a complex protein network, the presence and the amount of the principal, responsible proteins have been checked to provide an overview of the factors involved in cholesterol homeostasis in old rats.

It has been already reported that in aged rats, the hepatic HMG-CoAR, the rate-limiting enzyme of cholesterol biosynthesis, presents an unchanged level, but is fully active. Its degradation rate is slow and cholesterol synthesis is increased, while cholesterol content does not change in the liver but increases in the blood [Marino et al., 1998, 2002; Pallottini et al., 2003, 2004].

In an attempt to study the correlation between HMG-CoAR unchanged level and lowered degradation rate observed during ageing, the molecular basis of its long-term regulation have been assayed. The analysis of the Insigs, proteins deeply involved in reductase long-term regulation [Sever et al., 2003a,b] showed a significant reduction in accordance with the lower age-related HMG-CoAR degradation rate. In fact, the low amount of Insigs could not have been sufficient to bind the enzyme and induce its ubiquitination and degradation.

The relationship between Insig-1 level and insulin responsiveness in the ageing has been checked measuring the IRS phosphorylation, which represents the first signal of insulin cell responsiveness. The lower phosphorylated-IRS level found, confirmed all previous data [Fulop et al., 2003; Moller et al., 2003; Masoro, 2005], thus indicating that the reduction in insulin sensitivity could cause the decreased Insig-1 level and, in turn, the lowered HMG-CoA degradation rate. With regard to Insig-2, two isoforms are known: Insig-2a, which seems to be negatively regulated by insulin, and Insig-2b, which seems to be constitutive in the liver [Yabe et al., 2003]. In the absence of specific probes, it is impossible to determine which singular isoform is responsible for the slight reduction of Insig-2 observed in old rat liver.

Insigs are also involved in HMG-CoAR transcriptional regulation. Insig's bond to SCAP leads to ER retention of the SCAP/SREBP complex, which prevents the proteolytic generation of the transcriptionally active nSREBPs [Horton et al., 2002]. Our data show an increase in nSREBP-2, in accordance with Insigs reduced levels, and no significant variation in nSREBP-1 level. On the other hand, the increased SCAP levels, the reduced microsomal SREBPs levels, and the increase of nSREBP-2 suggest a large transfer of SREBPs to the Golgi apparatus. These results are interesting when considering that SREBP-2 is reported to preferentially activate the transcription of the genes related to cholesterol metabolism [McPherson and Gauthier, 2004], while SREBP-1 is retained to regulate the transcription of the genes related to fatty acid metabolism.

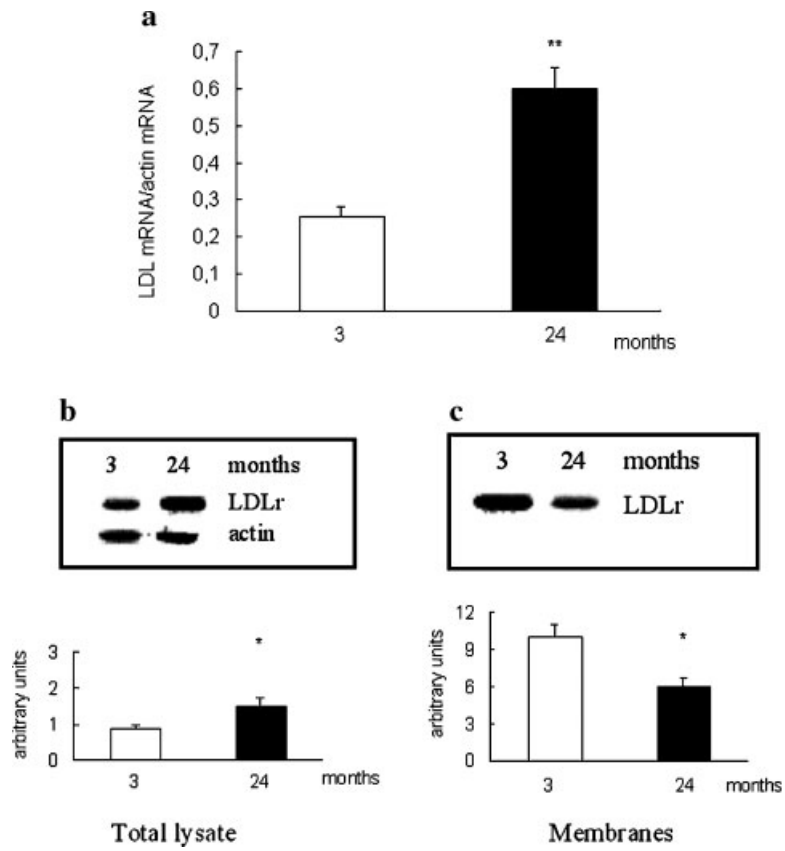

Fig. 6. LDLr mRNA and protein levels in adult and old rat liver. **Panel a** shows LDLr mRNA levels analyzed by real-time RT-PCR. Hepatic total RNA was reverse-transcripted and subsequently amplified by real-time PCR by using master mix with SYBR Green and sense and antisense primers for the tested gene (Table I). For details see the text. The bars represent the ratio between the number of LDLr mRNA molecules and the number actin mRNA molecules in old (24 months) rats and adult (3 months) rats. **Panel b** shows the LDLr protein levels in total lysate obtained as described in the Material and Methods section. **Panel c** shows the LDLr protein levels on membrane obtained as described in the Material and Methods section. Proteins from 20 μg membranes or total lysate were resolved by SDS–PAGE, subsequently transferred electrophoretically onto nitrocellulose and then probed with anti-LDLr antibody. The analysis was performed on four different experiments. $*P < 0.05$ as from a Student's *t* test with the respect to adult (3 months). $**P < 0.001$ as from a Student's *t* test with the respect to adult (3 months).

The detected increase in nSREBP-2 level should indicate an enhanced HMG-CoAR mRNA transcription in old animals, but on the contrary, the analysis of its mRNA level shows, a decrease. So both the lowered mRNA level and the lowered degradation rate of HMG-CoAR [Pallottini et al., 2004] could explain the unchanged protein level observed during ageing [Pallottini et al., 2003]. Therefore, we must assume that nSREBP-2 could be shifted to the transcription of some of the other SREBP-2 dependent genes. The higher amount of LDLr mRNA followed by the higher protein level detectable in the total cell lysate supports this assumption. However, the reduced membrane localization of LDLr could be related to the decreased Caveolin 1 amount measured on the membrane. This being so, the impairment of some cell processes committed to the membrane translocation (intracellular vesicular traffic) can not be excluded. Hence, the decreased LDLr membrane exposure and the following reduced LDL-cholesterol uptake could contribute to the hypercholesterolemia characteristic of aged rat as already reported by Marino et al. [2002] and confirmed in this study.

Age-related hormonal variation and sensitiveness induce a decreased ability to maintain homeostatic potential and are always associated with a modified content or functionality of some molecules. In particular, in this article it has been underlined that the decreased insulin sensitivity that occurs during ageing, induces changes of some factors involved in cholesterol metabolism, such as Insig-1 protein. Age-related Insigs reduction determines

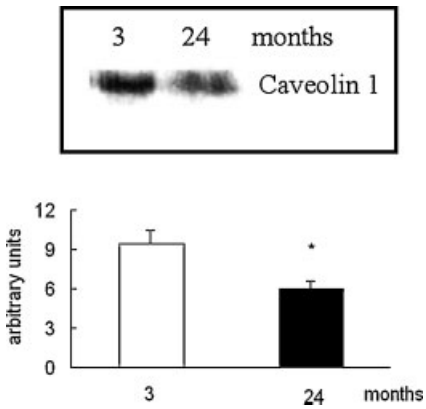

Fig. 7. Caveolin 1 protein level in adult and old rat liver. The figure represents on the top a typical Western blotting, on the bottom the densitometric analysis of the Caveolin 1 levels obtained from four different experiments. The experiments were performed in adult (3 months) and old (24 months) rat liver membranes. Proteins from 20 μg membranes were resolved by SDS–PAGE, subsequently transferred electrophoretically onto nitrocellulose and then probed with anti-Caveolin 1 antibody. For details see the text. $^*P < 0.05$ as from a Student's *t* test with the respect to adult (3 months).

the decreased degradation rate of the HMG-CoAR and the increase of nSREBP-2 even if mRNA amount transcripted of the analyzed nSREBP-2 dependent genes, increases in one cases, and decreases in another. Other SREBP-2 dependent genes behave differently in aged rats. In fact a reduced Caspase-2 mRNA level [Crott et al., 2004], or and increase of glucose-6-phosphate dehydrogenase and fatty acid synthase [Tollet-Egnell et al., 2004] has been described. At the moment, it is difficult to define the mechanism of these divergences. We can only hypothesize a different SREBP commitment to gene transcription or a different mRNAs half-life.

In conclusion, the observable hypercholesterolemia in aged rats is ascribable to the fully activated reductase and the lower LDLr membrane localization. The analysis of the levels of the main factors involved shows that Insigs, SREBPs, and SCAP undergo specific modifications that open a new scenery for their functional interplay at different stages of life.

TABLE II. Plasma Cholesterol Content in 3- and 24-Month Rat Liver

	3 Months	24 Months
Total-Chol (mg/dl plasma)	100 ± 12	415 ± 42**
HDL-Chol (mg/dl plasma)	45 ± 9	152 ± 19**
LDL-Chol (mg/dl plasma)	23 ± 8	75 ± 12**

Total Chol, HDL-Chol, and LDL-Chol in plasma of 3- and 24-month-aged rats. The data represent the mean ± SD of at least four different experiments. For details see the text.
$^{**}P < 0.001$ as from a Student's *t* test with the respect to adult (3 months).

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