Sex differences in hepatic regulation of cholesterol homeostasis

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Abstract

Physiological sex differences may influence metabolic status and then alter the onset of some diseases. According to recent studies, it is now well established that females are more protected from hypercholesterolemia-related diseases, such as cardiovascular diseases until menopause. Female protection from hypercholesterolemia is mediated by the hypolipidemic properties of estrogens, even if mechanisms underlying this protection remain still debated. Even though the regulatory mechanisms of cholesterol homeostasis maintenance are well known, few data are available on the supposed differences between male and female in these processes. So, the aim of this work was to define, through an *in vivo* study, the putative sex-dependent regulation of the processes underlying cholesterol homeostasis maintenance. We examined 3-hydroxy 3-methylglutaryl coenzyme A reductase and its regulatory protein network as well as the amount of low-density lipoprotein receptor and cholesterol. The study was conducted in the liver and plasma of male and female rats, on adults and during postnatal development, and on 17-β-estradiol-treated male rats. Our data support that physiological differences in proteins involved in cholesterol balance are present between the sexes and, in particular, 3-hydroxy 3-methylglutaryl coenzyme A reductase shows lower activity and expression in female and 17-β-estradiol-treated male rats than in adult untreated male. Our data suggest that sex differences in enzyme expression depend on variation in regulatory proteins and seem to be related to estrogen presence. This work adds new evidence in the complicated picture of sex-dependent cellular physiology and establishes a new role for reductase regulatory proteins as a link between estrogen protective effects and cholesterol homeostasis.

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Introduction

Recent research, over the past decade, has been dedicated to the study of the relevant physiologic differences between the sexes that may affect the prevention, diagnosis, and treatment of disease. Dissimilar exposures, susceptibilities, and responses to initiating agents, and differences in energy storage and metabolism result in variable responses to pharmacologic agents and the onset and manifestation of diseases such as obesity, autoimmune disorders, and coronary heart disease (Wizemann & Pardue 2001).

According to these gender studies, it is well established that females are more protected from hypercholesterolemia-related diseases, such as cardiovascular diseases (CVD) until menopause (Maxwell 1998). Female protection is principally mediated by estrogens, due to the hypolipidemic properties of these hormones (Barzel 1988, Farhat *et al.* 1996, Bär & Amelink 1997).

For instance, it has been established that lipoprotein profile among pre-menopausal women differs from men: low-density lipoprotein (LDL) cholesterol is lower while high-density lipoprotein (HDL) cholesterol is higher. After menopause, the LDL cholesterol becomes higher than age-matched males (Atkins *et al.* 2000). Women taking hormone replacement therapy show a reduction in LDL cholesterol levels (Skafar *et al.* 1997). The potential benefits of estrogens against CVD are not only related to lipoprotein profile improvement, but also estrogens appear to reduce the risk of these diseases through a combination of effects, including endothelial nitric oxide generation, cell proliferation and angiogenesis, and regulation of vascular smooth muscle cells Ca^{2+} and K^{+} channels. These effects may be mediated through genomic and/or non-genomic mechanisms exerted by female sex hormones (Skafar *et al.* 1997).

Moreover, studies on animal models of estrogen deficiency indicated that there is a disruption of lipid homeostasis; presumably, this is acting primarily through estrogen receptor α (Heine *et al.* 2000, Jones *et al.* 2000, Ohlsson *et al.* 2000).

Among lipids, cholesterol plays an essential function in cell membrane structure and fluidity maintenance and is the precursor of steroids and bile acids. Moreover, it is now known that disorders in cholesterol metabolism lead to human diseases.

Cholesterol homeostasis is maintained mainly by a feedback regulatory system that senses the level of cholesterol in cell membranes and modulates both the transcription of gene

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promoters encoding proteins of cholesterol biosynthesis as well as the uptake from plasma lipoproteins and post-transcriptional events (Brown & Goldstein 1999).

Two pathways provide mammalian cells with cholesterol: receptor-mediated endocytosis of LDL by LDL receptor (LDLr) and the new synthesis from acetyl-CoA via the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR; Espenshade & Huges 2007). Because of its pivotal role in cholesterol and nonsterol isoprenoid compound biosynthesis, most of the mechanisms involved in cholesterol homeostasis are related to HMG-CoAR long- and short-term regulation.

HMG-CoAR is an endoplasmatic reticulum (ER) membrane-spanning enzyme, catalyzing the four-electron reduction of HMG-CoA to CoA and mevalonate (MVA), which is then converted to isopentenyl pyrophosphate, the building block for cholesterol and nonsterol isoprenoids (Goldstein & Brown 1990, Pallottini *et al.* 2004).

Short-term regulation of the enzyme is operated by post-translational mechanisms such as phosphorylation and dephosphorylation of the catalytic domain, which render the enzyme respectively catalytically inactive or active. Specific kinases (mainly AMP-dependent kinase, AMPK; Ching *et al.* 1996) and phosphatases (mainly protein phosphatase 2A, PP2A; Gaussin *et al.* 1997) are responsible for such interconversion.

HMG-CoAR is physiologically present in the cell in unphosphorylated active form (30–40%) and phosphorylated inactive form (60–70%; Pallottini *et al.* 2004). The ratio between unphosphorylated form and total form indicates the activation state of the HMG-CoAR.

Long-term control of HMG-CoAR works on the synthesis and degradation rate of the enzyme.

Sterol regulatory elements (SREs) are nucleotidic sequences in the gene promoters, encoding proteins involved in cholesterol synthesis and homeostasis, such as HMG-CoAR and LDLr. These sequences are recognized by a family of transcription factors called SRE-binding proteins (SREBPs) that play a central role as regulators of cellular lipid homeostasis (Goldstein *et al.* 2006).

Two genes code for three SREBP isoforms, SREBP 1a, SREBP 1c, and SREBP 2 (Tontonoz *et al.* 1993, Song *et al.* 2005). SREBP 2 isoform preferentially regulates genes involved in cholesterol homeostasis including all the sterol biosynthetic enzymes, such as HMG-CoAR and LDLr (Horton *et al.* 2002). Once synthesized, SREBPs are carried to and associated with the ER membrane where they remain transcriptionally inactive (Bengoechea-Alonso & Ericsson 2007). In ER, the C-terminus of SREBPs interacts with a cargo protein called SREBP cleavage-activating protein (SCAP), which functions as a sterol sensor (Edwards *et al.* 2000, Goldstein *et al.* 2006). In sterol-depleted cells, SCAP escorts SREBPs from the ER to Golgi where proteolytic activation of SREBP occurs, this part of the protein (nSREBP) migrates into the nucleus encoding its target genes (Bengoechea-Alonso & Ericsson 2007).

Both SCAP and HMG-CoAR share a polytopic intramembrane sequence called the sterol-sensing domain (SSD; Espenshade & Huges 2007). Sterols are able to bind the SSD and modify the tertiary structure of the proteins. In the presence of sterols, the conformation adopted by these proteins allows them to bind to another ER protein: Insig that is able to regulate both the transcription and the degradation of HMG-CoAR (Epand 2006).

Hormonal regulation of HMG-CoAR is assured by the interplay of several hormones acting at different levels (Pallottini *et al.* 2004). Thyroid hormones, for example, regulate HMG-CoAR gene expression; glucocorticoids act at post-translational level (Geelen *et al.* 1986), while insulin has been reported to affect both transcriptional (Feramisco *et al.* 2004) and post-translational mechanisms (Leoni *et al.* 1985). In particular, available data about estrogen effects on HMG-CoAR regulation are yet controversial. Initially, a role for physiologic levels of estrogen as a positive effector of HMG-CoAR activity was reported by Carlson *et al.* (1980). However, other data show that pharmacological 17-β-estradiol treatment in male rats *in vivo* induces a decline in hepatic HMG-CoAR activity after 6 h and a rapid LDLr induction (Marino *et al.* 2001). Furthermore, other studies provide evidence that LDLr and HMG-CoAR are respectively up- and downregulated by 17-β-estradiol, this fact induces a growth inhibition or apoptosis in DLD-1 colon cancer cell line (Messa *et al.* 2005), and that estrogens are able to modulate LDLr transcription through a mechanism mediated by tyrosine kinase signaling in HepG2 cells (Distefano *et al.* 2002).

Few fragmentary and very old papers are present in literature about putative physiological sex differences in cholesterol homeostasis-related proteins (Abul-Hajj 1978, Carlson *et al.* 1980, Choi *et al.* 1988, Hewitt *et al.* 2003, Kojina & Degawa 2006), and overall none of them focused on the mechanism underlying. Thus, the aim of this work was to evaluate whether basic sex differences are observable in the hepatic proteins that regulate cholesterol homeostasis maintenance; in addition, whether or not these supposed differences could be ascribable to physiological estrogen effects has been studied.

Indeed, we investigated, through *in vivo* study, the putative different expression patterns of hepatic proteins involved in cholesterol balance in adult rats, male and female. Then, we evaluated the protein levels in males and females, 8- and 15-day old, representing two developmental stages where serum estradiol and testosterone levels in female and in male respectively are physiologically modified (Banu *et al.* 2002, Zapatero-Caballero *et al.* 2004). Finally, the direct estrogen effects were evaluated treating adult male rats with pharmacological doses of 17-β-estradiol, contributing to better understanding estrogen involvement in these mechanisms.

Materials and Methods

Materials

All materials used were obtained from commercial sources and of the highest quality available. All materials with no specified source were obtained from Sigma–Aldrich.

Animals

Male and female *Rattus norvegicus*, Sprague-Dawley (Morini, Italy), 8-day, 15-day, and 3-month old, were housed under controlled temperature (20±1 °C), humidity (55±10%), and light (from 0700 to 1900 h). Animals had free access to food and water. The experiments were performed according to the ethical guidelines for the conduct of animal research (Ministero della Salute, Official Italian Regulation No. 116/92, Communication to Ministero della Salute no. 391/121).

Three-month-old male rats were injected intraperitoneally with 1 mg/kg of 17-β-estradiol 3-benzoate (Sigma Chemical Co.) in vehicle (1 ml/kg dimethyl sulfoxide (DMSO) in saline). Control animals received an equal volume of vehicle. Sampling was therefore performed at 2 and 24 h after the estradiol injection.

All male and female rats were anaesthetized with ether in a fume cupboard. Plasma was obtained from blood collected into EDTA (1 mg/ml blood). Livers were removed and rapidly frozen in liquid nitrogen. Four animals per group were used and livers independently analyzed.

HMG-CoAR activity

The assay was carried out with the radioisotopic method, following the production of [^{14}C]-mevalonate from 3-[^{14}C]-hydroxymethylglutaryl coenzyme A (3-[^{14}C]-HMGCoA; specific activity 57·0 mCi/mmol; GE Healthcare, Little Chalfont, UK).

Microsomes were prepared in the presence or absence of NaF according to Bruscalupi *et al.* (1985). NaF was used to block lisosomal phosphatase thus permitting the analysis of expressed HMG-CoAR, while microsomes without NaF are useful to evaluate total HMG-CoAR activity. Microsomes (100 μg) were incubated in the presence of cofactors (20 mM glucose-6-phosphate, 20 mM NADPC sodium salt, 1 IU glucose-6-phosphate dehydrogenase, and 5 mM dithiothreitol). The assay, in a final volume of 200 μl, was started by the addition of 10 μl (0·088 μCi/11·7 nmol) of 3-[^{14}C]-HMGCoA. The radioactivity of the produced [^{14}C]-mevalonate, isolated by chromatography on AG1-X8 ion exchange resin (Bio-Rad Laboratories), was counted. An internal standard (3-[^{3}H]-mevalonate, specific activity 24·0 mCi/mmol; GE Healthcare) was added to calculate the recovery.

Protein levels analysis

Protein levels were analyzed by western blotting. Analysis of INSIG 1, INSIG 2, and HMG-CoAR was performed on microsomes prepared as described previously. Total lysates and total membranes were prepared according to Martini *et al.* (2007). Western blot analysis of LDLr was performed both on total membranes and total lysates prepared while the analysis of SREBP 2 (C-terminus) was performed on total lysates. Table 1 describes the sources and the secondary antibodies used.

Twenty micrograms of protein from solubilized membranes were resolved by 12% (for Insig 2) and 7% (for SREBP 2, LDLr and HMG-CoAR) 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% BSA in 138 mM NaCl, 27 mM KCl, 25 mM Tris–HCl, 0·05% Tween-20 (pH 6·8), and probed at 4 °C overnight with primary antibodies, then 1 h with the secondary ones. The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL, USA) for 10 min at room temperature and then probed with anti-tubulin antibody (MP Biomedicals, Solon, OH, USA). Bound antibodies were visualized using enhanced chemoluminescence detection (GE Healthcare).

All the images derived from western blotting were analyzed by ImageJ (NIH, Bethesda, MD, USA) software for Windows as arbitrary units. Each reported value was derived from the ratio between arbitrary units obtained by protein band and the respective tubulin band (chosen as housekeeping protein).

Hepatic cholesterol analysis

Fifty milligrams of liver tissue were homogenized in chloroform:methanol:H_2O 4:2:1 v/v. The mixture was

Table 1 List of antibodies used for western blotting assay

	Sources	Secondary antibodies	Sources
Primary antibodies			
Insig 1	Novus Biologicals, Littleton, CO, USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic, Rome, Italy
Insig 2	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit anti-goat conjugated to HRP	Chemicon International, Temecula, Canada
HMG-CoAR	Upstate, Lake Placid, NY, USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
SREBP 2	Abcam, Cambridge, UK	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
LDLr	Abcam	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Tubulin	MP Biomedicals, Solon, OH, USA	Goat anti-mouse conjugated to HRP	UCS Diagnostic

stirred on a vortex mixer for 2 min and then left for 15 min at room temperature. The samples were then centrifuged for 10 min at 600 ***g***. The chloroform fraction was transferred and dried under N_2, then the samples were dissolved in 100 ml ethyl ether and chromatographed on a thin layer chromatography Silica Gel 60 Å 20×20 (Whatman, Maidstone, England), previously activated at 100 °C for 60 min. Samples were developed in petroleum ether:ethyl ether:acetic acid 75:25:1 v/v; bands were visualized with iodine vapor and compared with standards (cholesterol).

Plasma cholesterol analysis

Plasma cholesterol content was assessed through colorimetric CHOD-POD kit according to manufacturer's instruction (Assel, Rome, Italy).

Statistical analysis

Each mean derives from four different experiments performed in duplicate. The statistical analysis was performed by ANOVA followed by Tukey–Kramer post test. *P* values under 0·05 were considered statistically significant. All the statistical analyses have been done using GraphPad Instat3 (Graphpad Software Inc., La Jolla, CA, USA) software for Windows.

Results

Sex differences in adult male and female rats

The first step in studying gender differences in HMG-CoAR regulation was the evaluation of the protein levels of the enzyme and proteins involved in its regulation, in the liver of adult male and female rats. As shown in Fig. 1a, protein levels in female liver are significantly lower than male.

HMG-CoAR activity is regulated by phosphorylation–dephosphorylation mechanisms, with the unphosphorylated form representing the active enzyme and the phosphorylated form the inactive. The expressed and the total enzyme activities were measured in microsomes prepared in the presence or absence of NaF respectively. It is interesting to note that the total −NaF activities is approximately fivefold lower in the liver of female than of male in agreement with low protein levels (Fig. 1b). No significant changes between sexes in the +NaF/−NaF ratio (r), which reflects the activation state of the enzyme *in vivo*, are observable, indicating that HMG-CoAR phosphorylation–dephosphorylation mechanisms should not be involved in these sex differences.

So, we focused our interest on HMG-CoAR long-term regulation-related proteins, such as Insigs and SREBP 2.

To check whether lower HMG-CoAR expression in females could be related to a lower amount of its main transcription factor, the SREBP 2 level was analyzed. The 125 kDa uncleaved protein is expressed on ER and nuclear membrane, while the transcriptionally active form (nSREBP 2) is represented by a 68 kDa protein. Figure 2a shows that both uncleaved SREBP 2 and nSREBP 2 are lower in female than in male.

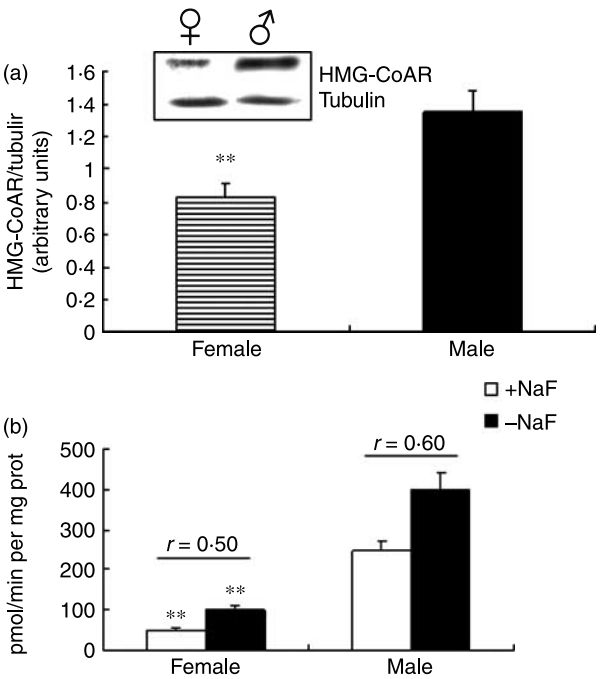

Figure 1 HMG-CoAR levels and activity in adult male and female rat liver. (a) A typical western blotting and the densitometric analysis of HMG-CoAR levels performed on four different male and female adult rat livers. The protein levels were normalized with tubulin content. (b) HMG-CoAR activity performed on four different male and female adult rat livers. White columns represent the activity in the presence of NaF (phosphatase inhibitor) and black columns represent the total enzyme activity calculated in the absence of NaF. The enzyme activity is expressed in pmol per min per mg proteins. The ratio between +NaF and −NaF activity represents the activation state of the enzyme (r). All the data obtained are the mean±S.D. of four different experiments in duplicate. $^{**}P<0{\cdot}001$ as from ANOVA followed by Tukey–Kramer test with respect to adult male liver.

Since INSIGs are the main proteins involved in HMG-CoAR transcription (via SREBP 2) and proteosomal degradation, the levels of these proteins have been evaluated. Data obtained show higher Insig levels in female than male, in agreement with the low level of both HMG-CoAR and nSREBP 2 (Fig. 2b).

To assess whether the observed differences in HMG-CoAR levels could be due to an end-product feedback regulation, we checked hepatic and plasma total cholesterol content and the total and membrane levels of the main protein involved in intracellular cholesterol uptake, the LDLr. Figure 3a and b shows that no differences between male and female are observable in liver and plasma cholesterol content as well as in hepatic LDLr total and membrane expression (Fig. 3c and d).

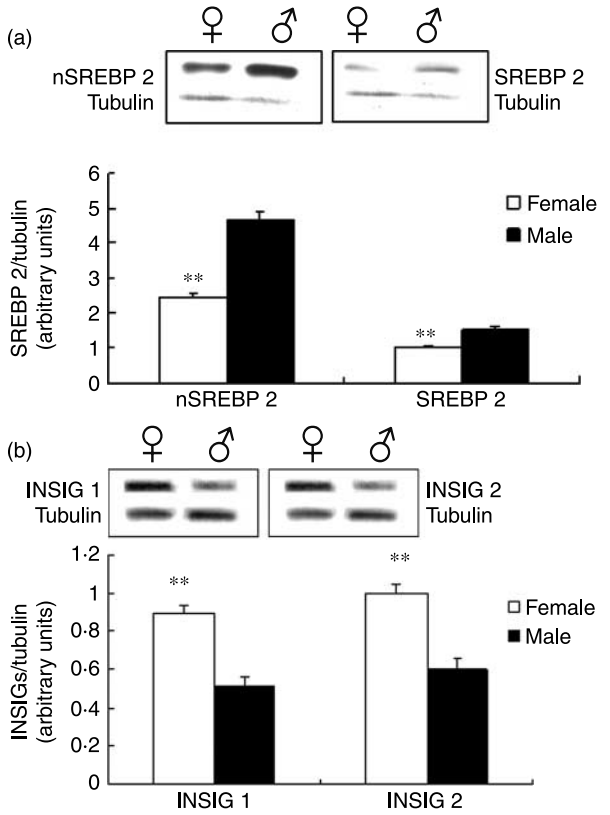

Figure 2 INSIG 1 and INSIG 2 levels and nuclear and total SREBP 2 levels in adult male and female rat liver. (a) A typical western blotting and the densitometric analysis of nSREBP 2 and SREBP 2 levels performed on four different male and female adult rat livers. INSIG 1 and INSIG 2 levels in male and female adult rat livers are illustrated in (b). The protein levels were normalized with tubulin content. All the data obtained are the mean ± S.D. of four different experiments in duplicate. $^{**}P<0{\cdot}001$ as from ANOVA followed by Tukey–Kramer test with respect to adult male liver.

Treatment of male rats with 17-β-estradiol

In order to investigate whether differences observed in adult animals could be associable with female sexual hormones, the effects in adult male rats treated for 2 and 24 h with pharmacological doses of 17-β-estradiol were studied. Data obtained by estrogen-treated male rats were compared with vehicle-treated male rats and adult male and female rats.

After 24 h (but not after 2 h) of estradiol treatment, treated male rats show a similar amount of HMG-CoAR to adult females, protein levels fall dramatically with the respect to adult males and vehicle control (Fig. 4a). HMG-CoAR activity in 24 h-treated males is comparable with adult females also, and there are no changes in the activation state of the enzyme (Fig. 4b).

Insig and SREBP 2 levels in 17-β-estradiol-treated rats were also studied. After 24 h of treatment, the levels of these proteins were comparable with adult female and completely different from adult male (Fig. 5a and b). LDLr levels were also

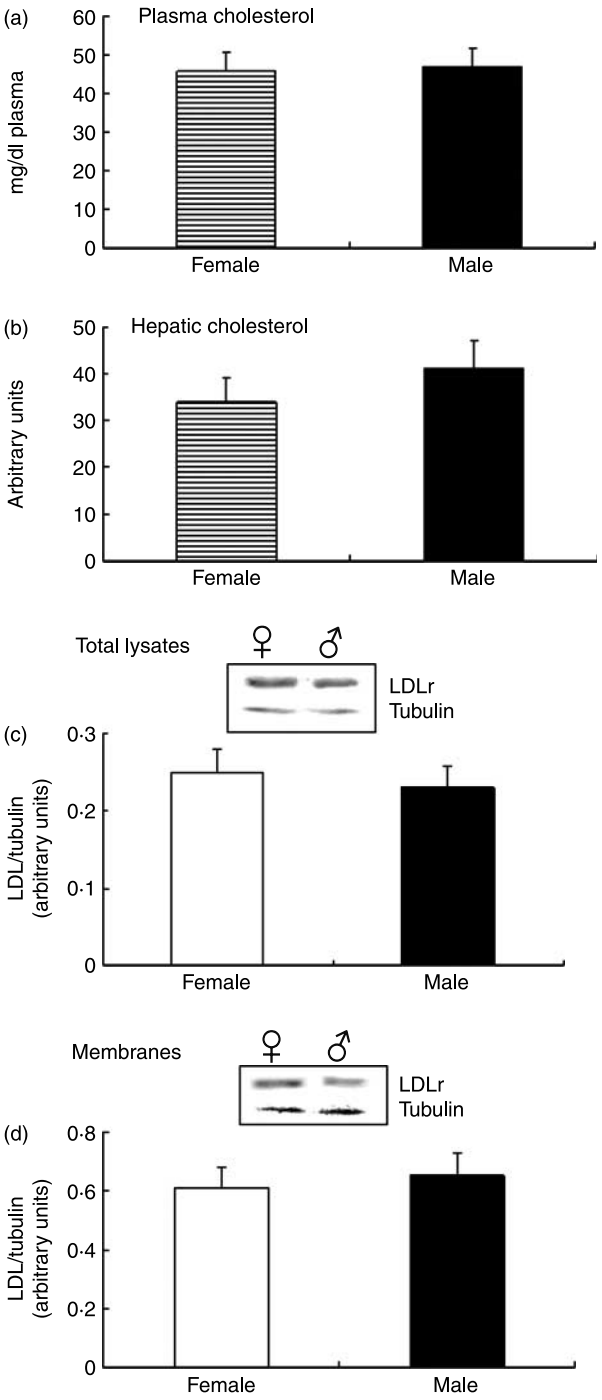

Figure 3 Liver and plasma cholesterol levels, total and membrane LDLr content in adult male and female rat liver. (a and b) Plasma cholesterol and hepatic cholesterol levels performed on four male and female adult rats are respectively represented. (c and d) A typical western blotting and the densitometric analysis of respectively LDLr total and membrane content from the livers of four adult male and four female rats normalized with tubulin amount. All the data obtained are the mean ± S.D. of four different experiments in duplicate.

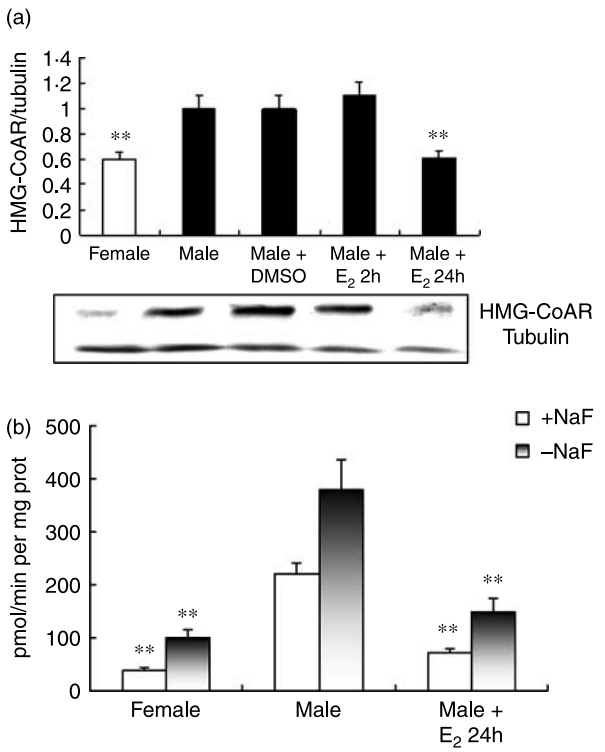

Figure 4 HMG-CoAR levels and activity in adult female, male, and 17-β-estradiol-treated adult male livers. (a) A typical western blotting and the densitometric analysis of HMG-CoAR levels obtained from four different livers of adult male and female rats, DMSO-treated males (placebo) and males treated with 17-β-estradiol 1 mg/kg (E_2) for 2 and 24 h, protein levels were normalized with tubulin. (b) Hepatic HMG-CoAR activity performed in the presence and absence of NaF (phosphatase inhibitor) in four different females, males, and 24-h E_2-treated males. The ratio between +NaF and −NaF activity represents the activation state of the enzyme (r). All the data obtained are the mean±S.D. of four different experiments in duplicate. $^{**}P<0{\cdot}001$ as from ANOVA followed by Tukey–Kramer test with respect to adult male liver.

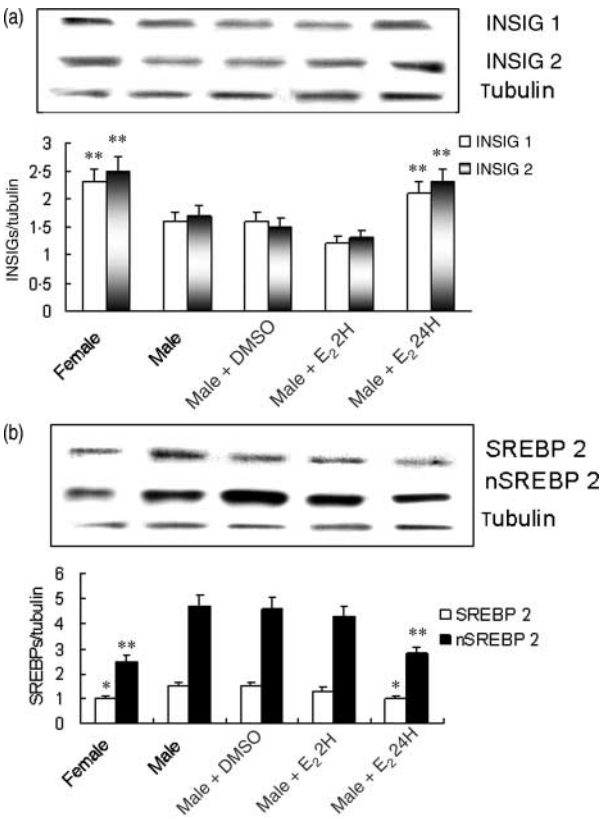

Figure 5 INSIG 1 and INSIG 2 and nuclear and total SREBP 2 levels in adult female, male, and 17-β-estradiol-treated adult male livers. (a) A typical western blotting and the densitometric analysis of Insig 1 and Insig 2 levels obtained from four different livers of adult males and females, DMSO-treated males (placebo) and males treated with 17-β-estradiol 1 mg/kg (E_2) for 2 and 24 h. (b) A typical western blotting and the densitometric analysis of nSREBP 2 and SREBP 2 levels obtained from four different livers of females, males, and 2- and 24-h E_2-treated males. Protein levels were normalized with tubulin. All the data obtained are the mean±S.D. of four different experiments in duplicate. $^{*}P<0{\cdot}05$ and $^{**}P<0{\cdot}001$ as from ANOVA followed by Tukey–Kramer test with respect to adult male liver.

evaluated (data not shown), but as already reported its expression after pharmacological 17-β-estradiol administration rises (Marino *et al*. 2001).

Sex differences during rat development

Because estrogen pharmacological treatment causes LDLr increase and could give rise to predictable both homeostatic and metabolic alterations, we chose two different stages of rat development where circulating sex hormone levels in male and female rats are physiologically modified. Eight-day-old rats show lower amounts of circulating sex hormones than 15-day ones similar to adult as already reported (Banu *et al*. 2002, Zapatero-Caballero *et al*. 2004). Also in these experimental models, HMG-CoAR and INSIG protein levels were studied.

HMG-CoAR levels do not change from 8 days to 3 months of age in male rats, while in females enzyme levels decrease starting from 15 days (Fig. 6a). The levels of INSIGs result to be higher in female than male rat liver starting from 15 days of age; at 8 days of age, there is a difference between the two isoforms, in fact INSIG 1 results to be lower in female than male and Insig 2 shows a opposite behavior (Fig. 6b).

Also in these experimental models, we analyzed LDLr levels to control a putative end-product feedback regulation but we did not find any sex differences in either group (Fig. 7).

Discussion

Understanding the molecular basis of the well-known physiological and morphological gender differences could provide new approaches in pharmacological treatment of various diseases. Phenotypes associated with sex are in

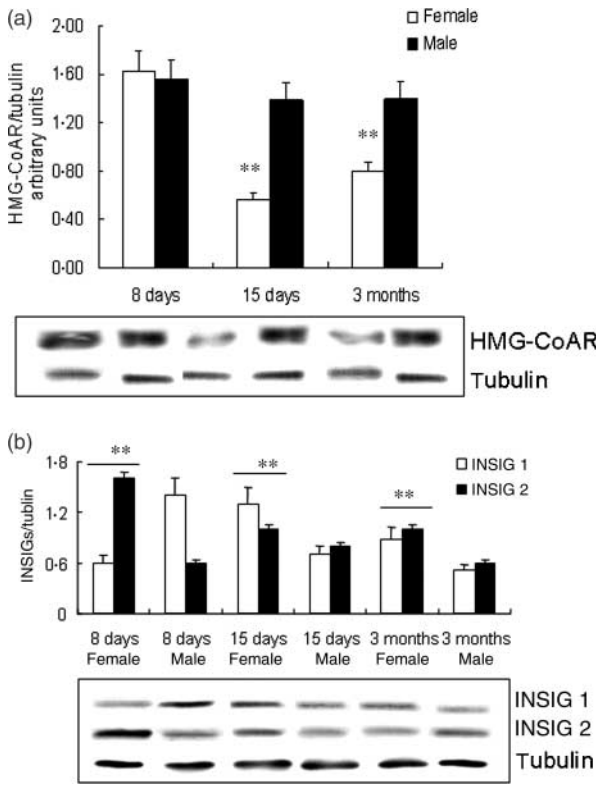

Figure 6 HMG-CoAR and INSIG 1 and INSIG 2 levels in the liver of 8-day-, 15-day-, and 3-month-old rat. HMG-CoAR levels performed on four different livers from 8-day-, 15-day-, and 3-month-old male and female rats are shown in (a) that illustrates a typical western blotting and the densitometric analysis. (b) INSIG 1 and INSIG 2 levels in four different livers of 8-day-, 15-day-, and 3-month-old male and female rats. Protein levels were normalized with tubulin content. All the data obtained are the mean ± S.D. of four different experiments in duplicate. $^{**}P<0{\cdot}001$ as from ANOVA followed by Tukey–Kramer test with respect to age-matched male livers.

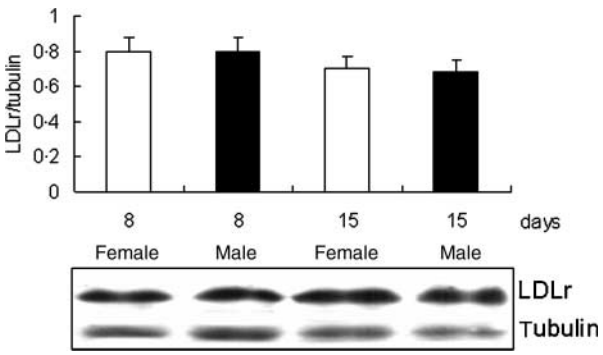

Figure 7 Membrane LDLr content in the liver of 8- and 15-day-old rat. The figure shows a typical western blotting and the densitometric analysis of LDLr membrane content obtained from four different livers of 8- and 15-day-old male and female rats, LDLr was normalized with tubulin amount. All the data obtained are the mean ± S.D. of four different experiments in duplicate.

turn associated with different risk in the onset of some diseases, such as CVD in which hypercholesterolemia plays a key role.

Even though the regulatory mechanisms of cholesterol homeostasis maintenance are well known (Espenshade & Huges 2007), few data are available on the supposed differences between male and female in these processes. So, the aim of this study was to define the putative sex-dependent regulation of the processes underlying cholesterol homeostasis maintenance. For this purpose, we examined the key enzyme of the cholesterol biosynthetic pathway, HMG-CoAR, and its regulatory proteins as well as the amount of LDLr and cholesterol. The study was conducted in the liver and plasma of male and female rats, during development and in adult phases, and on 17-β-estradiol-treated male rats.

Our data provide evidence that physiological differences in proteins involved in cholesterol balance are present between sexes; in particular, HMG-CoAR shows lower activity and expression in female and in 17-β-estradiol-treated male rats than in adult untreated male. The regulatory protein (SREBP 2, INSIGs) variations between sexes are functionally in agreement with HMG-CoAR behavior, according to the classical model well accepted (Goldstein *et al.* 2006, Espenshade & Huges 2007), while no differences are observable in either LDLr expression or the cholesterol amount that excludes an end-product feedback involvement in physiological estradiol presence.

The difference in the male and female expression patterns of HMG-CoAR and regulatory proteins seems to be related to estrogen presence. Our data suggest that sex differences in HMG-CoAR expression depends on variation in regulatory proteins. In particular, INSIG proteins could be the link between estrogen and HMG-CoAR.

Mammalian genomes contain two *INSIG* genes that code for two isoforms: INSIG 1 and INSIG 2, both of them are ER-resident proteins and they are functionally interchangeable, but the gene encoding Insig 1 is a nSREBP target and is induced by insulin; in contrast, the gene encoding Insig 2 isoform is not a nSREBP target and is constitutively expressed (Yabe *et al.* 2002, 2003). However, recent data show that in its promoter, the *INSIG* 2 gene presents some responsive boxes to transcription factors and nuclear receptors, such as the heterodimer retinoid X receptor–vitamin D receptor (RXR–VDR), peroxisome proliferator-activated receptors (PPARs), retinoic acid receptors (RARs; Lee *et al.* 2005), liver X receptor (LXR; Kim *et al.* 2003), and farnesoid X receptor (FXR; Hubbert *et al.* 2007).

INSIG proteins are able to drop cholesterol content through two parallel mechanisms. The first by inducing the degradation of HMG-CoAR and the second by blocking the transcription of the cholesterologenic enzymes as well as of the LDLr that is mainly involved in extracellular cholesterol uptake (Martini & Pallottini 2007).

Since INSIGs are the main proteins involved in HMG-CoAR levels regulation, the higher levels observed in females explain the low amount of HMG-CoAR. Moreover, alignment sequence analysis shows that the rat *INSIG* 2

gene presents an estrogen response elements-like (ERE-like) within intron 2, while many Half-ERE within rat *INSIG* 1 genes are observable (Ensembl rat genome data base, Sequence alignment program Clustal W).

Actually, low HMG-CoAR expression in females indicates that the neo-synthesis of cholesterol is physiologically lower than male and it could be related to the well-known evidence that females are more protected against hypercholesterolemia and hypercholesterolemia-related diseases until menopause. The lower HMG-CoAR activity in the female rat should mean a lower cholesterol synthesis and cholesterol content in the blood; on the contrary, the plasma cholesterol levels are not different between sexes. It is well known that the presence of estrogens markedly augment intestinal cholesterol absorption in animals and humans (Henriksson *et al.* 1989, Wang *et al.* 2004, Martini & Pallottini 2007), consequently balancing the minor synthesis, and this could explain the comparable amount of cholesterol in males and females. So, the observed protection from cholesterol-dependent diseases could be explained only by the already known different plasma ratio of LDL and HDL cholesterol (Heiss *et al.* 1980).

The first evidence for estrogen-related effects in these sex differences might be reasonably explained by observing protein behavior in 17-β-estradiol-treated male rats. Actually, 24-h treated male rats show the same HMG-CoAR and INSIG levels as female ones, indicating a genomic mechanism involvement; this hypothesis is supported by the 2-h treatment in which no protein level modifications are observable.

As pharmacological treatment could elicit no predictable both homeostatic and metabolic alterations, to confirm estrogen-related effects with the use of developmental rats was absolutely necessary. In fact, 15-day-old rats are characterized by a physiological burst of sex hormones that reach the same plasma concentration as adults (Banu *et al.* 2002, Zapatero-Caballero *et al.* 2004) and show an expression pattern of studied proteins comparable with that of adult rats. When testosterone peak is present (15-day old), no change in HMG-CoAR levels are observable in males since, in fact, 8-day- to 3-month-old HMG-CoAR levels do not change in males. On the contrary, when estradiol rises in female (15-day old), HMG-CoAR levels are reduced. The results obtained in animals subjected to such physiological stimulation support the hypothesis of 17-β-estradiol involvement in this regulation.

Notably, HMG-CoAR regulatory protein levels in 8-day-old rats are not functionally in agreement with the classical regulation model well known by the scientific community (Goldstein *et al.* 2006). This focus is under active investigation in our laboratories. One hypothesis could be that in this early phase of life the hormonal sensitivity of cells are not yet mature as already described (Leoni *et al.* 1985).

Thus, data obtained by the presented work add new evidence in the complicated picture of sex-dependent cellular physiology and establish a new role for INSIG as a link between estrogen protective effects and cholesterol homeostasis.

Declaration of interest

There are no conflict of interest that would prejudice the impartiality of this research.

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