Aging Is Associated With a Shift of Fatty Metabolism Toward Lipogenesis

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The incidence of nonalcoholic fatty liver disease is steadily increasing among the elderly population. Lipid metabolism is transcriptionally controlled by the nuclear receptors retinoid acid receptor alpha, liver-X-receptor alpha, and peroxisome proliferator–activated receptor alpha and their target genes ABCA1, sterol regulatory element–binding protein-1c, and fatty acid synthase. Using senescence-accelerated prone mice (SAMP8), we addressed the question as to whether age-related increase of oxidative stress affects nuclear receptor gene expression. In contrast to SAMR1 control mice, young SAMP8 mice exhibit hepatic steatosis with increased hepatic cholesterol content, plasma triglyceride, and aspartate aminotransferase levels. This is accompanied by an increase of liver-X-receptor alpha and retinoid acid receptor alpha expression, whereas peroxisome proliferator–activated receptor alpha expression is found diminished. SAMP8 mice further reveal a lower expression of ABCA1 as well as of sterol regulatory element–binding protein-1c and higher expression of fatty acid synthase. The dysbalance between the nuclear receptors and their target genes most probably mediates hepatic steatosis and underlines the pathological relevance of nuclear receptor shift toward lipogenesis in fat metabolism of the elderly patient.

Key Words: Senescence—Nuclear receptors—Hepatic steatosis.

Received February 4, 2011; Accepted June 30, 2011

Decision Editor: Rafael de Cabo, PhD

The incidence of nonalcoholic fatty liver disease is steadily increasing among the elderly population, not least due to age-related oxidative stress (1). The diagnosis of nonalcoholic fatty liver disease requires the evidence of fatty changes in the liver in the absence of excessive alcohol consumption. Hepatic steatosis as the hallmark of nonalcoholic fatty liver disease is accompanied by an excessive intrahepatic triglyceride content. Steatosis is defined chemically as intrahepatic triglyceride content >5% of liver volume or liver weight (2) or histologically when 5% or more hepatocytes contain visible intracellular triglycerides (3).

The progressive increase of oxidative stress during aging not only causes oxidative damage to cellular macromolecules but also modulates the pattern of gene expression (4) probably including fat metabolism regulating nuclear receptors. Among others, these nuclear receptors are liver-X-receptor alpha (LXRα) and 9-cis retinoid acid receptor alpha (RXRα), which mediate fat metabolism by lipid sensing, synthesis, and oxidation (5). LXRα is an important regulator of cholesterol, fatty acid, and glucose homeostasis (6). Through binding of endogenous LXR agonists 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 27-hydroxycholesterol (oxysterols oxidized cholesterol derivates), LXRα forms a heterodimer with the obligate partner RXRα (7,8) and regulates gene expression via LXR response elements in the promoter regions of its target genes (9).

Among others, these target genes are the ATP-binding cassette transporter ABCA1 (member 1 of human transporter subfamily ABCA), which mediates the efflux of cholesterol, the fatty acid synthase (FAS), which catalyzes the synthesis of fatty acids from acetyl-coenzyme A and malonyl-coenzyme A, and the sterol regulatory element–binding protein-1c (SREBP-1c), which regulates genes of the cholesterol metabolism (10–12). Beside LXRα and RXRα, the peroxisome proliferator–activated receptor alpha (PPARα), a further nonsteroidal nuclear receptor, is known to heterodimerize with RXRα and to bind to the peroxisome proliferator hormone response element (13,14). PPARα functions as a "sensor" for free fatty acids and regulates the lipid metabolism by inducing the lipolysis due to the increase of β-oxidation in peroxisome, which in turn leads to a decrease of systemic as well as hepatic triglycerides and cholesterol concentrations (15). Thus, PPARα can be considered as an antagonist of the lipogenic LXR. The balance of lipolysis and lipogenesis may be altered in the aging organism and therefore might predispose for the age-related hepatic steatosis. Based on this hypothesis, we used the senescence-accelerated prone mouse (SAMP8) and the corresponding control mouse, that is, the senescence-resistant mouse (SAMR1) as...
experimental approach. Beside age-associated pathologies such as neurodegenerative diseases, various skin lesions, hearing impairment, cataract, or immune deficiency (16–18), the SAMP8 mouse strain has been shown to exhibit severe liver pathology (19), increased oxidative stress (18,20–22), and high systemic triglyceride levels (19). Thus, this model seems to be ideally suited to study the potential mechanism underlying age-related changes in fat metabolism and to address the contribution of nuclear receptors (23).

METHODS

Animal Model

Female SAMP8 and SAMR1 mice were provided by Harlan and were used at 2, 6, and 12 months of age. Animals were kept on water and standard laboratory chow ad libitum. All animals received humane care according to the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23 revised 1985).

Sampling and Assays

All animals were exsanguinated by puncture of the vena cava inferior for immediate separation of plasma, followed by harvest of liver tissue. Plasma aspartate aminotransferase activity was measured spectrophotometrically as indicator for hepatocellular disintegration and necrosis. Measurement of plasma triglycerides, serving as an indicator of dyslipidemia, was performed using the triglyceride assay kit method according to the manufacturer’s instructions (Cayman Chemical Company, MI).

For measurement of hepatic cholesterol content, lipids were extracted by means of the Bligh–Dyer method (24). Briefly, livers were incubated with a mixture of chloroform and methanol (1:2). After vortexing, one part of chloroform and one part of H2O was added and spun down by 3,000g to separate phases. The organic phase (lower layer) was collected and concentrated by vacuum pump. The cholesterol content was analyzed by using the cholesterol and/or cholesteryl ester quantitation kit method according to the manufacturer’s instructions (Calbiochem, Merck KGaA, Darmstadt, Germany).

RNA Analysis of Liver Tissue

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed with SuperScript First Strand Synthesis System (Invitrogen) in accordance to the manufacturer’s instructions.

Real-time quantitative polymerase chain reaction assays were performed by using Lightcycler 1.5 and the Lightcycler FastStart DNA MasterPlus SYBER GreenI kit (Roche Diagnostics GmbH, Germany). Each amplification mixture (20 μL) contained 5 μM primer, 19 μL of universal polymerase chain reaction Mastermix, and 1 μL 1:2 diluted complementary DNA solution. Polymerase chain reaction thermocycling parameters were 95°C for 10 minutes and 40 cycles of 95°C for 10 seconds, 55°C for 5 seconds, and 72°C for 10 seconds. All samples were analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. For analysis of the relative change in gene expression, we used the 2-ddCt method. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of polymerase chain reaction amplification. The following mouse primers were used: RXRα forward (5′-AGC CAT TGT CCT GTT CAA CC-3′), RXRα reverse (5′-TCC ATG AGG AAG GTG TCG AT-3′), LXRα forward (5′-TGCCATCAGCATTCTCTTG-3′), LXRα reverse (5′-GGCTCACCAGCTTTACAG-3′), PPARα forward (5′-GGAAGGCTTTCTGTGACATC), PPARα reverse (5′-TCATCTGAGTGTCTCTG-3′), ABCA1 forward (5′-ACTGGAGACACCCCTGTGAC-3′), ABCA1 reverse (5′-GGAGACTTTCCGTTTGGTCG-3′), SREBP-1c forward (5′-GTA CCT GCG GGA CAG CTT AG-3′), SREBP-1c reverse (5′-CAG GTC ATG TTG GAA ACC AC-3′), FAS forward (5′-TACCATGGCAACGTGACT-3′) FAS reverse (5′-TAGCCCTCCGGACACTC-3′), GAPDH forward (5′-CGTCCCCAGACAAATGTG-3′), and GAPDH reverse (5′-GAATTGCGTGAGTGGAGT-3′).

Western Blot Analysis of Liver Tissue

Harvested liver tissue was further processed for protein isolation. For this purpose, liver tissue was homogenized in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, 0.5% Triton-X 100, 0.02% NaN3, and 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail), incubated for 30 minutes on ice, and centrifuged for 10 minutes at 4°C and 10,000g. Protein contents were assayed by bicinechonic acid method (Pierce Biotechnology) with bovine serum albumin (Pierce Biotechnology) as standard. On 12% sodium dodecyl sulfate gels, 40 μg protein was separated and transferred to a polyvinylidifluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). After blockade with 2% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA), membranes were incubated overnight at 4°C with a mouse monoclonal anti-RXRα (clone F-1, 1:2,000, Santa Cruz), a rabbit polyclonal anti-LXRα/β (clone H-144X, 1:5,000, Santa Cruz), a goat polyclonal anti-PPARα (clone N-19, 1:2,000, Santa Cruz), and a rabbit polyclonal anti-SREBP-1c (clone C-20; 1:500, Santa Cruz) antibodies, respectively. Afterward, a secondary peroxidase-linked rabbit anti-mouse (RXRα; 1:60,000; Sigma), donkey anti-goat (PPARα; 1:20,000, Santa Cruz), and goat anti-rabbit antibodies (LXRα/β, 1:15,000, SREBP-1c, 1:10,000; Cell Signaling) was applied. Protein expression was visualized by means of
luminol-enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and digitalized with ChemiDoc XRS System (Bio-Rad Laboratories, Munich, Germany). Signals were densitometrically assessed (Quantity One; Bio-Rad Laboratories) and normalized to the β-tubulin signals (rabbit polyclonal anti-β-tubulin antibody; 1:500; Santa Cruz).

Histology

For hematoxylin and eosin staining and immunohistochemical analysis of RXRα, hepatic tissue was fixed in 4% phosphate-buffered formalin for 2–3 days and subsequently embedded in paraffin. From the paraffin-embedded tissue blocks, 4-μm thin sections were put on poly-l-lysine–covered glass slides and exposed to a mouse monoclonal anti-RXRα antibody (F-1, 1:100, Santa Cruz). For the development of RXRα with 3,3′-diaminobenzidine chromogen, Universal LSAB kits (System-HP; DakoCytomation, Dako, Hamburg, Germany) were used according to the manufacturer’s instructions. The sections were counterstained with hemalaun and analyzed with a light microscope (Olympus BX51, Hamburg, Germany). For planimetric analysis of RXRα, positive nuclei images of 20 randomly selected low-power fields (×10 magnification, Olympus BX 51) were acquired with a Color View II FW camera (Color View, Munich, Germany) and evaluated by means of an image analysis system (Adobe Photoshop, Adobe Systems, Uxbridge, UK).

Statistical Analysis

All data are expressed as means ± standard errors of the mean. Statistical differences were determined using analysis of variance, followed by post hoc pairwise comparison tests for analysis between either strains or stages of life. Data were considered significant if \( p < .05 \). Statistical analysis was performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA).

Results

Hepatic Steatosis During Aging

Livers of SAMP8 mice showed a progressive deposition of microvesicular fat droplets in hepatocytes, with most pronounced vacuolization at the age of 12 months (Figure 1). Of note, 2-month-old SAMP8 mice exhibited already significantly higher content of hepatic cholesterol than SAMR1 mice. Hepatic cholesterol content further doubled with the age of 12 months in SAMP8 mice and constantly exceeded the respective values of the SAMR1 mice (Figure 2A). Furthermore, SAMP8 mice demonstrated a 1.8-fold increase of systemic triglyceride concentrations (Figure 2B) and a 2.1-fold increase of aspartate aminotransferase plasma activity (Figure 2C) when compared with age-matched SAMR1 mice. SAMR1 control mice showed an age-related increase of both triglycerides and aspartate aminotransferase levels with values at the age of 6 and 12 months being in the range of those of SAMP8 mice (Figure 2B and C).

Impact of Aging on Nuclear Receptor Protein Expression

To evaluate nuclear receptors in dependency to age, we first determined the protein expression of RXRα, LXRα, and PPARα in 2-, 6-, and 12-month-old SAMR1 and SAMP8 mice. Immunohistochemical and Western blot analysis showed that aging of mice was associated by a
progressive and significant rise of RXRα protein expression (Figure 3A–C), with values in SAMP8 mice slightly but constantly exceeding those of SAMR1 mice (Figure 3A–C). Comparable results were found by Western blot analysis of LXRα with an age-dependent increase of LXRα protein expression in both mouse strains (Figure 4A and B). On the contrary, the Western blot analysis of PPARα showed a moderate decrease of PPARα protein expression in both mouse strains with lowest values in the 12-month-old SAMP8 mice (Figure 4C and D). Of utmost interest, expression of SREBP-1c, a target gene of LXRα/RXRα, was significantly reduced in SAMP8 mice already at the age of 2 months and at all further time points studied, whereas expression of SREBP-1c in SAMR1 mice decreased age dependently, however, with values being constantly above those of SAMP8 mice (Figure 4E and F).

Impact of Aging for Nuclear Receptors and Their Target Gene Messenger RNA Level

After confirmation that the expression of GAPDH is not changed with age, GAPDH served as housekeeping gene. In contrast to protein expression, messenger RNA (mRNA) expression of RXRα decreased from 2 to 6 months of age in livers of both mice strains and slightly increased at the age of 12 months (Figure 5A). Thereby, SAMP8 mice revealed a moderately lower RXRα mRNA expression as age-matched SAMR1 mice (Figure 5A). LXRα mRNA expression analysis revealed significantly higher expression levels at 6 months of age in SAMR1 versus SAMP8 mice (Figure 5B). Livers of SAMP8 mice showed a very slight increase of LXRα mRNA expression with age (Figure 5B). In contrast to protein expression of PPARα, the mRNA expression increased from 2 to 6 months of age in livers of both SAMR1 and SAMP8 mice. At the age of 12 months, hepatic PPARα mRNA expression was found decreased to levels corresponding to those found in 2-month-old mouse livers (Figure 5C). In general, SAMP8 exhibited lower levels of hepatic PPARα mRNA expression than SAMR1 mice (Figure 5C).

Target gene mRNA analysis confirmed the fact that SAMP8 mice showed the feature of hepatic steatosis in that livers of SAMP8 mice exhibited at all time points studied a significantly lower ABCA1 mRNA expression compared with livers of SAMR1 mice (Figure 5D). Furthermore, mRNA expression of SREBP-1c was found significantly lower in 2-month-old SAMP8 mice versus age-matched SAMR1 mice (Figure 5E). With progressive aging, the mRNA expression of SREBP-1c also decreased in SAMR1 mice with thus statistically not differing values between the two mouse strains at the age of 6 and 12 months (Figure 5E). FAS mRNA expression was found progressively increasing with aging in livers of both mouse strains (Figure 5F). 12-month-old SAMP8 and SAMR1 mice revealed a three- and fivefold increase when compared with their corresponding 2-month-old mice (Figure 5F). At 12 month of age, SAMP8 mice exhibited almost twofold higher FAS mRNA expression than SAMR1 mice (Figure 5F).

Figure 2. Analysis of hepatic cholesterol content (A), plasma triglycerides (B), and plasma aspartate aminotransferase (AST) activity (C) in 2-, 6-, and 12-month-old SAMR1 (n = 6) and SAMP8 (n = 6) mice. Values are given as means ± standard errors of the mean; analysis of variance, post hoc pairwise comparison tests: *p < .05 vs age-matched SAMR1 mice, #p < .05 vs 2-month-old mice of the identical genetic background.
The major findings of the present study are that aging in SAMP8 mice is accompanied by hepatic steatosis, as well as increased hepatic cholesterol content, and plasma triglyceride and aspartate aminotransferase levels. Of interest, the expression of the nuclear receptors LXRα and RXRα is increasing with age in both mouse strains, whereas PPARα expression is exclusively diminished in SAMP8 mice. Furthermore, lower expression of both ABCA1 and SREBP-1c as well as higher expression of FAS in SAMP8 mice imply that aging is associated with a shift of fatty metabolism toward lipogenesis.

A demographic change in population is often accompanied with an increase of the metabolic syndrome (25), comprising multiple deteriorations of liver function. Not least due to age-related oxidative stress the incidence of hepatic steatosis is rising (26). In line with this, the senescent-accelerated prone mouse is characterized by an increased oxidative stress (17) and shows an age-dependent degeneration of the liver (19). Thus, it is obvious that SAMP8 mice develop hepatic steatosis. Indeed, we could show that SAMP8 mice exhibit higher hepatic cholesterol content with accompanying hypertriglyceridemia and marked vacuolization of the hepatocytes, indicative for organ steatosis. Thereby, vacuolization is characterized by cytoplasmatic fat droplets, which press the hepatocellular nucleus to the cell membrane (27). Beside steatosis, livers of SAMP8 mice undergo a certain extent of damage, as given by the increased transaminase levels, similarly as already described by Ye and colleagues (19).

Age-related dysfunctions of fat metabolism and consequently an accumulation of triglycerides and cholesterol lead to a dysbalance of lipid homeostasis. It is assumed that defects of nuclear receptors due to age-related oxidative
stress may be causative for the dysbalance between lipolysis and lipogenesis. In this study, we found three key regulators of lipid metabolism, LXRα, RXRα, and PPARα, to be expressed in the hepatic tissue of SAMP8 and SAMR1 mice. Moreover, our experiments showed that LXRα/RXRα heterodimers also displayed DNA-binding activity (data not shown). It is known that LXRα acts as sensor for cholesterol and regulates lipogenesis (6,7). In turn, high

Figure 4. Representative Western blot and densitometric analysis of liver-X-receptor alpha (A and B), of peroxisome proliferator–activated receptor alpha (C and D), and of sterol regulatory element–binding protein-1c (E and F) in liver tissue specimen of 2-, 6-, and 12-month-old SAMR1 (n = 6) and SAMP8 (n = 6). Values are given as means ± standard errors of the mean; analysis of variance, post hoc pairwise comparison tests: *p < .05 vs age-matched SAMR1 mice.
cholesterol content initiates activation of LXR\(\alpha\) (9). The present study now demonstrates that SAMP8 mice exhibit higher cholesterol content in liver tissue, though the expression of LXR\(\alpha\) is almost comparable in both mouse strains. This underscores the view that not the expression but rather the activation of nuclear receptors is essential for regulation of

Figure 5. Quantitative real-time polymerase chain reaction analysis of the nuclear receptors retinoid acid receptor alpha (A), liver-X-receptor alpha (B), and peroxisome proliferator–activated receptor alpha (C), as well as of their target genes ABCA1 (D), sterol regulatory element–binding protein-1c (E), and fatty acid synthase (F) in liver tissue specimen of 2-, 6-, and 12-month-old SAMR1 \((n = 6)\) and SAMP8 \((n = 6)\) mice. Signals were corrected to that of glyceraldehyd-3-phosphate dehydrogenase. Values are given as means ± standard errors of the mean; analysis of variance, post hoc pairwise comparison tests: * \(p < .05\) vs age-matched SAMR1 mice, # \(p < .05\) vs 2-month-old mice of the identical genetic background.
the lipid metabolism. One might expect an accelerated increase of LXRα in aged SAMP8 mice due to the higher cholesterol content. However, SAMP8 and SAMR1 mice did not markedly differ in LXRα and RXRα expression at the age of 6 and 12 months. This might be due to the fact that SAMR1 mice show a “normal” aging process (20,28), with, thus, the consequence that SAMP8 mice lose their senescence-prone phenotype at higher ages.

Beside LXRα and RXRα, PPARα, which regulates lipolysis by activation of fatty acid β-oxidation, is capable to compensate an age-related increase of lipid accumulation. Both nuclear receptors LXRα and PPARα heterodimerize with RXRα and activate transcription of their target genes via specific response elements (29). It is supposed that LXRα and PPARα are competing for a limited pool of RXRα (30). Thus, the increase of PPARα mRNA expression in livers of 6-month-old SAMP8 and SAMR1 mice might in turn be causative for the decrease of RXRα mRNA expression in these livers. However, at the age of 12 months, LXRα/RXRα expression was found slightly increased with a concomitant decrease of PPARα expression, forwarding the heterodimerization of LXRα/RXRα and thus the switch of lipid homeostasis toward lipogenesis. This may lead to an accumulation of lipids with the organism losing its ability to compensate arising triglycerides (31) and to prevent the development of hepatic steatosis. This theory seems to be particularly valid in SAMP8 mice, as the dysbalance of factors regulating lipid homeostasis, that is, decreased PPARα expression, is more pronounced. In line with this, Vilà and colleagues (23) could demonstrate that SAMP8 mice exhibit hypertriglyceridemia and decreased β-oxidation, which might be causative for the pronounced reduction of PPARα expression.

It has been shown by the present study and data of others (5,30,32) that aging is characterized by an increase of LXRα-dependent FAS expression, which leads to an activation of the de novo fatty acid synthesis. Due to this fact and the knowledge that saturated fatty acids inhibit the expression of ABCA1 (33), it is obvious that—despite comparable LXRα expression in both mouse strains—the ABCA1 expression is suppressed in the SAMP8 mice. In addition, unsaturated fatty acids antagonize the activation of LXRα and thus suppress the transcription of SREBP-1c (34). SREBP-1c regulates the genes responsible for cholesterol metabolism, including ABCA1. Thus, the decreased expression of SREBP-1c in SAMP8 mice might also explain the reduced expression of ABCA1. The third explanation for reduced ABCA1 expression in SAMP8 mice can be deduced from the fact that PPARα upregulates ABCA1 expression (6). Less PPARα expression, as observed in SAMP8 mice of the present study, might be associated with reduced ABCA1 expression. Ouvrier and colleagues (35) demonstrated that a lack of ABCA1 causes an accumulation of cholesterol, a mechanism which might also account for the high cholesterol content in the present SAMP8 mice.

For attenuation of systemic triglycerides and cholesterol levels, clofibrates and fenofibrates such as Lipanthyl are applied that activate PPARα and therefore induce lipolysis (15). In addition, LXR agonists are used for the therapy of hypertriglyceridemia and arteriosclerosis (36,37). However, they are associated with side effects such as overstimulation of hepatic lipogenesis (9). Because aging is still disregarded with respect to the development of new drugs, the current finding, that is, fatty metabolism is shifted toward lipogenesis with age, provides an interesting and promising target for the development of new therapeutic strategies.

Acknowledgment
The authors cordially thank the technicians of the Institute for Experimental Surgery, University of Rostock, for their valuable assistance.

References