Lifelong Caloric Restriction Reprograms Hepatic Fat Metabolism in Mice

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Calorie lowering slows the aging process and extends life span in diverse species by so far unknown mechanisms. The inverse linear relationship between calorie intake and life span suggests that regulators of energy metabolism are of importance in aging. The present study shows that lifelong caloric restriction in mice induces a metabolic adaptation with reduced lipogenesis and enhanced lipolysis and ketogenesis. This process, that is, the reprogramming of hepatic fat metabolism, is associated with a marked rise of fibroblastic growth factor 21 as a putative starvation master regulator. Due to the life span–extending properties of fibroblastic growth factor 21, the rise in fibroblastic growth factor 21 might contribute to the markedly better health status found in mice upon lifelong caloric restriction feeding. In addition, adropin, known as a peptide that controls lipid homeostasis, is significantly upregulated, underlining the diminution of lipogenesis that was further substantiated by decreased expression of liver-X-receptor α and its target genes sterol regulatory element–binding protein-1c, fatty acid synthase, and member 1 of human transporter subfamily ABCA upon lifelong caloric restriction feeding.

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In all species studied to date, restricting calorie intake by 20%–50% while still providing adequate micronutrients significantly extends mean and maximal life span (1,2). There is intense interest in elucidating the molecular mechanisms underlying the antiaging effects of caloric restriction (CR) (3,4). Most recently, it is demonstrated that a growth factor, namely the fibroblastic growth factor 21 (FGF21), is capable of extending life span (5). FGF21, abundantly expressed in the liver (6), is a putative starvation master regulator (5). According to this, it could be shown that expression of FGF21 is induced in fasted mice or in mice exposed to a ketogenic diet (7). However, in response to a short-term CR the expression of FGF21 does not increase (5). Further, it is known that FGF21 is a direct target gene of the peroxisome proliferator-activated receptor-α (PPARα) in mouse liver (7–9). In turn, PPARα functions as a “sensor” for free fatty acids and regulates the lipid metabolism by inducing lipolysis via increased β-oxidation in peroxisome, which leads to a decrease of both systemic and hepatic triglyceride as well as cholesterol concentrations (10). An antagonist to PPARα is the liver-X-receptor α (LXRα), which forms a heterodimer with the obligate partner 9-cis retinoid acid receptor α (RXRα) (11,12) and regulates gene expression via LXR response elements in the promoter regions of its target genes (13). Among others, target genes are (i) the adenosine triphosphate-binding cassette transporter ABCA1 (member 1 of human transporter subfamily ABCA), which mediates the efflux of cholesterol, (ii) the fatty acid synthase (FAS), which catalyzes the synthesis of fatty acids from acetyl-CoA and malonyl-CoA, and the (iii) sterol regulatory element–binding protein-1c (SREBP-1c), which regulates genes of the cholesterol metabolism (14–16). The balance between lipolysis and lipogenesis is altered in the aging organism (17) and therefore might predispose to a fat accumulation with age. In response to this, leptin, an adipokine, which is secreted in proportion to body fat stores (18), is upregulated. Leptin is involved in the control of energy balance and body composition (19) by regulating both energy intake and expenditure (20). It is reported that CR causes a substantial decrease of plasma leptin concentrations (21,22), suggesting a role for this hormone during the CR-induced metabolic adaptation (23) most probably by the activation of the PPARα pathway (24). A further peptide which regulates energy homeostasis is adropin. This peptide is secreted mainly by the liver and alters the expression of genes that are involved in lipogenesis (25). Until now, it is unknown whether and, if yes, how adropin is influenced by CR.

Based on the fact that aging is characterized by a shift of fatty metabolism towards lipogenesis (17), we hypothesized that the metabolic adaptation due to CR leads to reduced lipogenesis and enhanced lipolysis and ketogenesis. We focused our study on the impact of a lifelong calorie lowering, because most studies investigated the effect of short-term CR (26–28).
Methods

Animals
Sixty female C57BL/6 mice (Charles River Wiga, Sulzfeld, Germany) were used for this study. The mice were housed in standard cages in groups of five animals in a temperature-controlled room (22 ± 2°C) on a 12-hour light/dark cycle (light on at 06:00 AM) with free access to food and water under specific pathogen-free conditions. At the age of 4 weeks mice (average body weight 15.07 ± 0.61 g) were fed either ad libitum (AL) or CR (60% of AL) for four additional weeks (n = 10 for each group), 20 weeks (n = 10 for each group), or 74 weeks (n = 10 for each group). The mice were weighed weekly and directly before sacrifice. After transverse laparotomy, images of the 74-week AL or CR mice were taken to document the fat storage. The experimental protocol was approved by the local Animal Research Committee (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei [LALLF]) of the state Mecklenburg-Western Pomerania (LALLF M-V/TSD/7221.3-1.1-064/08), and 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).

Feeding
Mice were fed AL with pelleted standard laboratory chow (Ssniff R/M-H 10 mm, Soest, Germany; cat. no. V154-000) and tap water and served as reference groups. Food intake of five AL-fed mice housed per cage was measured once a week. From the daily eaten lab chow per AL-fed mouse the CR-fed mice received 60% once a day at 08:00 AM. Caloric restriction lasted for 4, 20, or 74 weeks.

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. Measurements of low-density lipoprotein/very LDL (LDL/VLDL) fraction, triglycerides, leptin, adiponectin, FGF21, and ketone bodies in plasma were performed using the LDL/VLDL cholesterol, leptin, adiponectin, and β-hydroxybutyrate assay kit methods according to the manufacturer's instructions (LDL/VLDL: Abcam, Cambridge, UK, leptin, adiponectin: Bachem/Peninsula Laboratories LLC, Torrance, CA, FGF21: R&D System, Minneapolis, MN, triglycerides, β-hydroxybutyrate: Cayman Chemical Company, Ann Arbor, MI). For measurement of hepatic cholesterol concentrations, lipids were extracted by means of the Bligh–Dyer method (29). 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/cholesterol 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 with the manufacturer’s instructions. Total RNA of 2 μg 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 Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). Each amplification mixture (20 μl) contained a 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 ribosomal protein S18 (RPS18) expression. For analysis of the relative change in gene expression we used the 2ΔΔCt method. A complementary DNA pool of livers of untreated C57BL/6J mice served as the control and therefore as the first A. The second A is represented by Ct-values of RPS18 amplification. 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: LXRα, forward (5′-TGCCCATCAGCATCTTCTCTG-3′), reverse (5′-GGCTCACAGCTCATTTATGC-3′); PPARα, forward (5′-GGAAGCGGTTCGTCGACATC-3′), reverse (5′-TCATCTGGATGGTTGCTCTG-3′); RXRα, forward (5′-AGCCATTTGCTTTGTCAC-3′), reverse (5′-TCCA TGAGAAAGTGTCTGAC-3′); ABCA1, forward (5′-ACT GGAGACACCCCTGTGA-3′), reverse (5′-GGAGACT TTCGT'TTGTTCG-3′); FAS, forward (5′-TACCATGGCA CGTGACACT-3′), reverse (5′-TAGCCCTCCCGTGACACT CAC-3′); SREBP-1c, forward (5′-GATCTGGGCGGACA GCTTAG-3′), reverse (5′-CAGGTATGTTGGAAACC AC-3′); FGF21, forward (5′-GCTGTCTTCCTGCTG GG-3′), reverse (5′-CCTGGTTTGGGAGGATCTTTC-3′); and RPS18 forward (5′-AGATGTAGATGAGATGGA AG-3′) and reverse (5′-TTGGATACCCACAGTTCG-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% Na3, 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 bicinchoninic 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) and a goat polyclonal anti-PPARα (clone N-19, 1:2,000; Santa Cruz), respectively. Afterwards, a secondary peroxidase-linked rabbit anti-mouse (RXRα; 1:60,000; Sigma) and a donkey anti-goat (PPARα; 1:20,000; Santa Cruz) 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-actin signals (mouse monoclonal anti-β–actin antibody; 1:20,000; Sigma).

Histology and Immunohistochemistry

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 either stained with hematoxylin and eosin or exposed to a mouse monoclonal anti-RXRα antibody (F-1, 1:100; Santa Cruz). For the development of RXRα with DAB chromogen Universal LSAB kits (System-HRP, 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). Images were acquired with a Color View II FW camera (Color View, Munich, Germany).

Statistical Analysis

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

RESULTS

Lifelong CR Slowed Increase of Body Weight

In general, survival rate and health status were better in the CR-fed group. All CR-fed mice reached the scheduled end of the experiments, whereas in the AL-fed group only 7 of 10 mice lived for 78 weeks. Moreover, CR-fed mice were smaller in body size and had fewer external age-related signs, as showing denser coat and barely grayed hair than the AL-fed mice. The body weight of 74 weeks AL-fed mice continuously increased twofold with aging up to 30.53 ± 0.98 g. Lifelong CR resulted in a very slow increase of body weight and reached values of 18.87 ± 0.39 g. In general, 4, 20, and 74 weeks CR-fed mice showed significantly lower levels of body weight compared with AL-fed mice (Figure 1A). Furthermore, CR (Figure 1C) mice exhibited no fat storage in contrast to AL mice that showed clearly visceral fat accumulation (Figure 1B).

Lifelong CR Reduced Hepatic and Systemic Lipid Accumulation

We examined the amount of hepatic cholesterol, a parameter reflecting lipogenesis. For a period of 4–74 weeks hepatic cholesterol significantly increased from 29 to 85 mM in AL-fed mice (p < .05 vs 4 wk AL-fed mice), but only from 44 to 67 mM in CR-fed mice (not significant vs 4 wk CR-fed mice; Figure 2A). Furthermore, a lifelong CR caused a significant decrease of systemic triglyceride concentrations (p < .05 vs 74-wk AL-fed mice; Figure 2B) and systemic LDL/VLDL fraction (Figure 2C). In line with this, livers of 74 weeks AL-fed mice showed a progressive deposition of microvesicular fat droplets in hepatocytes (Figure 2D), whereas livers of 74 weeks CR-fed mice exhibited no lipid accumulation (Figure 2E). To better characterize an amelioration of liver function upon lifelong CR the plasma activity of aspartate aminotransferase was measured. Analysis demonstrated that mice which received a 74 weeks CR exhibited a twofold reduction of aspartate aminotransferase plasma activity compared with 74 weeks AL feeding (p < .05; Figure 2F).

Lifelong CR Decreased Leptin but Increased Adropin Concentrations

Plasma concentrations of leptin, an adipokine secreted in proportion to body fat stores, continuously increased in AL-fed mice, as indicated by an almost threefold rise in 74 weeks AL-fed mice when compared with 4 weeks AL-fed mice (Figure 2G). In contrast, CR caused in dependency to duration of the diet a drastic decrease of plasma leptin concentrations with almost nondetectable levels at 20 and 74 weeks (Figure 2G). Further, we evaluated adropin, which is described as regulator of hepatic lipid metabolism. Plasma adropin levels remained unchanged in AL-fed mice at all time points of analysis (Figure 2H). Unlike AL mice, mice that were fed with CR for 4 weeks showed significantly decreased adropin concentrations. Lifelong CR resulted in a marked rise of adropin concentrations to fivefold higher values in 74 versus 4 weeks fed animals (p < .05; Figure 2H).
Figure 1. Body weight, g, (A) of 4 wk (n = 20), 20 wk (n = 20), and 74 wk (n = 17) ad libitum (AL) and caloric-restricted (CR) mice. Compared with the age-matched AL-fed mice, the CR-fed mice had significantly lower body weights. Values are given as mean ± SEM; analysis of variance, post hoc pairwise comparison tests: *p < .05 versus AL. After transverse laparotomy, images of 74-wk AL (B) and CR (C) fed C57BL/6J mice were taken, showing that, in general, CR mice exhibited much less visceral fat than the age-matched AL mice.

Figure 2. Analysis of hepatic cholesterol content (A) as well as plasma concentrations of triglycerides (B), low-density lipoprotein/very LDL (LDL/VLDL; C), aspartate aminotransferase (AST; F), leptin (G), plasma adipon (H), and representative light microscopic images (D and E, original magnification x400) of hematoxylin and eosin stained liver tissue. Mice were fed either ad libitum (AL) or caloric-restricted (CR, 60% of AL) for 4 wk (n =20), 20 wk (n = 20), or 74 wk (n = 17). Values are given as means ± SEM; analysis of variance, post hoc pairwise comparison tests (A, G, H): *p < .05 versus AL; *p < .05 versus 4 wk; $p < .05; Mann–Whitney U-test (B, C, and F): *p < .05 versus AL-fed mice.
Lifelong CR Diminished Lipogenesis and Increased Lipolysis

In AL-fed mice, the messenger RNA (mRNA) expression of LXRα increased in 20-week AL-fed mice and again decreased in 74-week AL-fed mice towards values found in 4-week AL-fed mice (Figure 3A). In contrast, lifelong CR lowered lipogenesis, as indicated by a constant decline of LXRα mRNA expression with values in 20- and 74-week CR-fed mice, which were far below those found in AL mice (Figure 3A). RXRα mRNA expression increased two to threefold in both AL and CR mice from 4 to 74 weeks of feeding, however with 38%–65% lower values in CR versus AL mice (Figure 3B). In line with this, immunohistochemical and Western blot analysis showed that a lifelong CR was associated with a significant decrease of RXRα protein expression in 74 weeks CR-fed mice as given in Figure 3D and E (p < .05 vs 74-wk AL-fed mice; Figure 3C and E). AL feeding resulted in an age-related reduction of lipolysis, as PPARα mRNA expression decreased by approximately 60% from 4 to 74 weeks (Figure 3F). In contrast to that, PPARα mRNA expression in 4 weeks CR-fed mice was low (p < .05 vs 4 wk AL-fed mice), but remained unchanged or even slightly increased over duration of diet (Figure 3F). In confirmation to that, Western blot analysis of PPARα demonstrated that livers of 74 weeks CR versus AL-fed mice expressed 30% more of PPARα protein (Figure 3G).

SREBP-1c, FAS, and ABCA1 increased in 20 weeks AL-fed mice and again decreased in 74 weeks AL-fed mice towards or even below values found in 4 weeks AL-fed mice (Figure 4A–C). In contrast, lifelong CR lowered lipogenesis, as indicated by a constant decline of mRNA expression of SREBP-1c, FAS, and ABCA1 with values in 20 and 74 weeks CR-fed mice, which were far below those found in AL mice (Figure 4A–C).

Although expression of FGF21 mRNA and systemic FGF21 protein were found almost unchanged over time...
in AL-fed mice, lifelong CR induced a 24-fold or 28-fold increase of FGF21 mRNA expression and systemic FGF21 protein concentration (p < .05 vs 4 wk CR-fed mice; Figure 4D and E). In line with this, CR-fed mice revealed a continuous rise of ketone bodies, as given by a fourfold increase of plasma β-hydroxybutyrate concentrations up to 0.55 mg/L in 74 weeks fed mice compared with 4 weeks CR-fed mice (p < .05; Figure 4F). On the contrary, the concentrations of β-hydroxybutyrate remained almost unchanged in AL-fed mice averaging at low values of 0.06 mg/L (Figure 4F).

**DISCUSSION**

The main finding of the present study is that in response to lifelong CR the organism increases lipolysis and ketogenesis. In addition, lipogenesis is strongly diminished by lifelong CR, which is reflected by decreased leptin, triglycerides, LDL/VLDL, and hepatic cholesterol amount, leading to an amelioration of liver function and structure. The mRNA expression of the nuclear receptor LXRα decreased with lifelong CR, whereas the RXRα and PPARα expression is maintained and accompanied by a compensatory rise of FGF21 mRNA expression and FGF21 protein content. Furthermore, the reduction of SREBP-1c, FAS, and ABCA1 mRNA expression during the course of the diet supports the view that lifelong CR is able to restore the age-related dysbalance of fat metabolism.

It is well documented, and the present study confirms, that aging is characterized by an increase of LXRα-dependent FAS expression, which leads to an activation of the de novo fatty acid synthesis and consequently results in an acceleration of lipogenesis (17,30–32). Thus, the demographic change in population rises the incidence of metabolic syndrome (33), comprising multiple deteriorations of liver function. However, epidemiological studies revealed that appropriate treatment, such as CR, can reduce or prevent obesity (34). Thus, it is essential to adequately establish such lifestyle factor during childhood and perpetuate them during the whole life in order to prevent metabolic diseases. Takemori and coworkers (35) demonstrated that a short-term CR increased PPARα expression with enhancement of β-oxidation, inhibition of triglyceride synthesis and therefore improvement of liver function. Our data now show that mRNA expression of PPARα was markedly lower upon 4-week short-term CR.
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References

LIFELONG CALORIC RESTRICTION FORCES LIPOLYSIS

Kumar and colleagues (25) described adropin as a peptide, which controls the lipid homeostasis, possesses the property to operate the saturation and downregulates the expression of lipogenic genes (25). Thus, the lifelong CR-induced increase of adropin may additionally protect the liver against an age-associated fat accumulation.

In summary, lifelong CR rescues the age-related dysbalance of fat metabolism towards lipolysis and ketogenesis and increases FGF21 expression, both most probably contributing to the improved health status at advanced age.

Feeding but remained unchanged or even slightly increased with duration of feeding, implying the maintenance of lipolysis upon lifelong CR. These data could be substantiated by the fact that a CR with duration of 74 weeks increased PPARα protein expression up to 30%. We further show that genes which are involved in lipogenesis are downregulated with lifelong CR. The mRNA and partially protein expression of both LXRα and RXRα and their target genes, like SREBP-1c, FAS, and ABCA1, are decreased, leading to an enhancement of the de novo fatty acid synthesis and probably to a deceleration of lipogenesis in response to lifelong CR. In support of these findings in CR-fed mice, AL-fed mice exhibited higher hepatic cholesterol content with concomitant hepatocellular vacuolization as sign of enhanced lipogenesis. However, CR mice showed also—though not significant—an increase of hepatic cholesterol amount. Because, it is known that fasting is associated with an increase of the hepatic cholesterol concentration (36), it can be assumed that also upon CR the synthesis of cholesterol is intensified in other organs due to reinforced lipolysis. Cholesterol will be then taken up from the blood stream to the liver to be metabolized directly via lipolysis (36).

FGF21 plays an important role in adaptation to metabolic states, which requires increased fatty acid oxidation (37). In line with the fact that mRNA expression of FGF21 is upregulated in vitro in HepG2 cells being exposed to acetocacetate (38) and in vivo in ketogenic diet fed mice (7), the current study shows a parallel rise of both FGF21 mRNA expression and β-hydroxybutyrate in lifelong CR-fed mice. This response can be interpreted that lifelong CR reprograms fat metabolism towards lipolysis and reflects the necessity to switch the hepatic metabolism towards ketogenesis as energy source (7). Another positive effect of FGF21 is the life-altering activity, because it is reported that FGF21-overexpressing mice show extended life span (5). Although FGF21 mRNA expression and in consequence the release of FGF21 protein did not increase during short-term (4 and 20 wk) CR, we could demonstrate that mice that received lifelong CR expressed higher levels of FGF21 mRNA and FGF21 protein. Because CR-fed mice exhibited a better health status and also an improved survival, it is conceivable to state that these mice benefited from the systemic secretion of FGF21 protein.

Beside nuclear receptors, adipocytokines, such as leptin, also regulate lipid metabolism (23,28). Leptin signaling is supposed to be involved in the antiaging effects of CR (24). Leptin secretion is primarily dependent from body adipose levels, and fasting plasma leptin concentrations are positively correlated with the percentage of body fat (24). This phenomenon was also observed in the present study, where in mice receiving a lifelong CR the plasma leptin concentrations were markedly decreased. In order to regulate saturation, adropin concentrations might be compensatory upregulated in the lifelong CR-fed mice. A study from
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