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SHORT-TERM CALORIE RESTRICTION AND REFEEDING DIFFERENTLY AFFECT LIPOGENIC ENZYMES IN MAJOR WHITE ADIPOSE TISSUE DEPOTS OF YOUNG AND OLD RATS

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The metabolic effects of short-term calorie restriction (SCR) and subsequent refeeding were compared in different white adipose tissue (WAT) depots of young (5-month old) and old (24-month) male Wistar rats. The animals were subjected to a 40% calorie restricted diet (*i.e.* 40% lower food supply than of control rats) for 30 days, and then re-fed for 0, 2, or 4 days. WAT samples from perirenal (pWAT), epididymal (eWAT), and subcutaneous (sWAT) depots were analysed for the enzymatic activities of ATP-citrate lyase (ACL), fatty acid synthase (FAS), and glucose-6-phosphate dehydrogenase (G6PD). The total WAT mass almost doubled in old rats, however, aging did not alter the relative proportions of the major regional fat depots. Serum leptin concentration was prominently higher in old rats, in which SCR resulted in less suppression of leptin level than in young animals, whereas refeeding increased leptin concentration in young, but not old, rats. In young rats refeeding elevated leptin gene expression only in pWAT, while in old rats the expression was induced first in eWAT, and later in pWAT. A prominent age-related decrease of ACL and FAS activities, but not of G6PD activity, was found in all the studied WAT depots. In young control rats, ACL activity was highest in pWAT, FAS activity was similar in all WAT depots, and G6PD activity was lowest in eWAT. In old rats, the enzymatic activities were lower in eWAT than in the other depots. The patterns of response to SCR and refeeding varied by age and WAT location. SCR stimulated ACL activity in pWAT but not in other depots of young rats, while FAS activity in pWAT and sWAT did not change in young and decreased in the old animals. Among the studied depots, pWAT was most responsive to refeeding in both age groups. In conclusion, SCR in old rats, as compared to the young, may be accompanied by reduced 'rebound effect' upon returning to unrestricted diet.

Key words: *short-term calorie restriction, aging, white adipose tissue depots, refeeding, lipogenic enzymes, leptin, glucose-6-phosphate dehydrogenase, fatty acid synthase*

INTRODUCTION

Aging in humans and other mammals is associated with the failure to maintain homeostasis in response to physiological and environmental challenges. Disturbances of fuel and energy homeostasis in aging occur in three major forms: glucose intolerance and type-2 diabetes, increased accumulation of fat leading to obesity, and, paradoxically, anorexia of aging resulting in the decrease of body mass or even cachexia (1). In all these conditions, there is a clear imbalance between the intake of energy substrates and their usage, which may arise from age-dependent changes in the neurohormonal regulation of energy homeostasis (2) and the reciprocal dysregulation of local metabolism in peripheral tissues (3). In both animals and humans, aging is associated with gender-dependent accumulation and redistribution of the adipose tissue (4, 5). In effect, a significant proportion of the elderly present elevated body weight with substantially increased fat mass, accompanied by decreased lean-body mass, all of which pose health risks. Moreover, aging promotes the accrual of fat in visceral depots, while subcutaneous depots gradually become depleted (6, 7). Such redistribution may either be a manifestation or a cause of metabolic impairments or diseases, such as insulin resistance, metabolic syndrome, and atherosclerosis (6).

In order to reduce body weight while preserving lean body mass, very low calorie diets (VLCD) which provide less than 50% of the predicted resting energy expenditure (8), and low calorie diets (LCD) have been introduced (9). These calorie-restricted diets are also frequently implemented in the elderly for the duration of several weeks (8), with varying efficacy in relation to body-weight reduction after returning to normal diet. Since, for ethical reasons, the metabolic consequences of such low-calorie diets are difficult to determine in humans on the tissue level, animal models of short-term calorie restriction (SCR) have been introduced. However, in contrast to the well-known effects of lifelong calorie restriction (LCR) in rodents and non-human primates such, as increased mean and maximum lifespan (in rodents), and delays in the onset of age-associated pathologies (10-12), fewer studies have described the impact of late-onset SCR on the metabolic and hormonal milieu in old animals (13-15). In rodents, as in many elderly people (4, 16), aging is associated with a prominent increase in white adipose

tissue (WAT) mass (5, 17). Since different structural and functional features characterize various WAT locations (7), we decided to assess the effects of short-term dietary restriction and subsequent refeeding on the lipogenic enzymes' activities in major WAT depots (perirenal, epididymal, and subcutaneous, *i.e.* pWAT, eWAT and sWAT, respectively) in young and old male Wistar rats. We measured the activity of three enzymes: fatty acid synthase (FAS), which catalyses the biosynthesis of long-chain saturated fatty acids from acetyl-coenzyme A and malonyl-coenzyme A, in the presence of NADPH; ATP-citrate lyase (ACL), which provides acetyl-coenzyme A; and glucose-6-phosphate dehydrogenase (G6PD), which is a major source of NADPH. Our findings provide novel observations on the effects of SCR and subsequent refeeding on the lipogenic enzymes' activities in major WAT depots of young and old rats.

MATERIAL AND METHODS

Animals

Male Wistar-Han rats, derived from the Charles River breed, were kept in the Academic Animal Experimental Centre in Gdansk at 22°C under a 12/12h light/dark cycle in specific pathogen-free (SPF) facility. Littermates were divided into pairs according to similar weight and food consumption, and subsequently caged by twos. No aggressive behaviors were noted throughout the experiment's duration. The rats' health was monitored weekly by a qualified veterinarian. Mortality of the Wistar-Han strain increases after 21 months of age (18). During the experiment two animals with palpable tumors were euthanized. Autopsy was also performed on each sacrificed experimental rat, however, no tumors or other gross pathology were detected. Animals were handled according with the Medical University of Gdansk's institutional guidelines for animal care and European Union laws. Experimental procedures were approved by the Local Ethical Committee (protocols no. 8/2009 and 16/2009).

Both young-adult (5-month old) and old (24-month) rats were divided randomly into two groups. The control animals (n=8, young and old rats, each group) were allowed free access to food and water. The other animals (CR, calorie restricted groups, n=26 and n=24, young and old rats, respectively) were allowed free access to water, and for 30 days were fed 60% of the total amount of food consumed by the control group (*i.e.* 40%-restricted diet). The food was supplied to all rats every day 2 hours after lights-on. The average daily food intake of the control group was measured as the difference in weight between the food provided and the food remaining (including spilled chow), and was 25.0±1.26 g and 20.0±0.84 g for the *ad libitum* fed young and old rats, respectively, over the duration of the experiment. The composition of the commercial diet (Labofeed H, Wytwornia Pasz Morawski, Kcynia, Poland; 52% of metabolic energy from carbohydrates, 11% from lipids, 37% from proteins) used in all groups was the same as that described in (19). After 30 days of short-term calorie restriction (CR), rats from both age groups were randomly divided into three subgroups: (a) 8–10 rats that were directly sampled after 30 days of CR; (b) 8 rats that were fed *ad libitum* for 2 days (CR+2 group); and (c) 8 rats that were fed *ad libitum* for 4 days (CR+4 group).

After treatment, rats were fully anaesthetized by intraperitoneal injection of ketamine and xylazine, blood was collected from the heart, and the animals were sacrificed by decapitation. Fat depots (perirenal, epididymal, and subcutaneous white adipose tissue harvested from the inguinal fossae) were sampled, weighted, immediately frozen in liquid nitrogen, and stored at –80°C until analysis. The rest of the fat tissue from each location was carefully excised and weighted.

Chemicals

All chemicals used, unless otherwise specified, were from Sigma-Aldrich (St. Louis, MO, USA).

Measurements of metabolite concentration in blood serum

Blood samples were allowed to clot and were subsequently centrifuged (2000 *g*, 15 min, 4°C). Sera were frozen at –80°C until analysis. Measurements of blood serum metabolite concentrations were performed in the Department of Clinical Biochemistry, Medical University of Gdansk, using routine diagnostic tests.

Serum leptin concentration

The concentration of leptin in the rat blood serum was measured by a species-specific ELISA test (Merck Millipore, St Charles, MO, USA), following the manufacturer's instruction.

Leptin gene expression

Frozen WAT samples were homogenised with zirkonia beads in the MagNALyser tissue homogeniser (Roche Diagnostics, Indianapolis, IN, USA). Total cellular RNA was isolated using the Total RNA Prep Plus extraction kit (A&A Biotechnology, Gdynia, Poland). First strand cDNA was synthesised from 1 g of total RNA using the M-MLV enzyme (Promega, Madison, WI, USA) and oligo$(dT)_{18}$ primers. Relative quantification of *Lep* mRNA expression was performed by real-time quantitative PCR (qPCR) with the housekeeping *β-actin* and *36B4* genes as internal controls. The cDNA matrices were amplified in SybrGreen PCR buffer (A&A Biotechnology, Gdynia, Poland) containing 0.2 µM sense and antisense primers. The primers used were:

Lep: 5'- GCTGCAAGGTCCAAGAAGAAGAA-3' and 5'-TGCCTGGCGGATACCGACT-3';

β-actin: 5'-GAAATCGTGCGTGACATTAAG-3' and 5'-GCTAGAAGCATTTGCGGTGGA-3' (20);

36B4: 5'-CTCAGTGCCTCACTCCATCA-3' and 5'-GGGGCTTAGTCGAAGAGACC-3' (21).

Amplification was carried out in StepOne Plus thermal cycler (Applied Biosystems, Carlsbad, CA, USA), in duplicate for each sample. Relative quantities of transcripts were calculated using the $2^{-\Delta\Delta CT}$ formula (22).

Measurements of enzymatic activities

0.2 g of tissue placed in 2 ml of ice-cold Tris buffer (25 mM Tris-HCl pH 7.8, 0.2% Triton X-100) was homogenized with a Teflon pestle homogenizer, and centrifuged at 30,000 *g* at 4°C for 20 minutes. The supernatant was used for enzyme assays. The enzymatic activities of ATP-citrate lyase (ACL, EC 4.1.3.8), fatty acid synthase (FAS, EC 2.3.1.85), and glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) were measured according to the modified methods described previously.

Briefly, ACL activity in WAT was assessed by the rate of NADH oxidation (absorbance at 340 nm) for 10 minutes at 37°C, using a Beckman DU 68 spectrophotometer (Beckman Instruments, Fullerton, CA, USA), as described in (23, 24). Determination of FAS activity in WAT homogenates was performed by measurement of the absorbance change at 340 nm due to NADPH oxidation. A correction was made for the rate of background NADPH oxidation in the absence of malonyl-CoA (24, 25). G6PD activity was assessed by measurement of NADP reduction at 340 nm. Because NADP reduction in this assay results from the activity of both G6PD and 6-phosphogluconate dehydrogenase, the activity of G6PD alone was determined by measuring the absorbance upon the addition of 5 mM glucose-

6-phosphate and subtracting the value from the previous result (23, 24).

Enzymatic activity was normalized to the total protein content of the tissue samples, measured according to Peterson's method (26).

Statistical analysis

Statistical analyses were performed using Statistica v.9 (StatSoft, Cracow, Poland). Data are presented as means ±S.E.M. Because not all sets of data followed normal distribution (Shapiro-Wilk test), nonparametric tests were used. The Mann-Whitney rank-sum test was used for comparisons of groups, while comparisons between different WAT depots were performed with Wilcoxon signed-rank test. Correlation analysis was determined using the Spearman test.

RESULTS

Effects of calorie restriction and refeeding on body mass and mass of white adipose tissue depots

Body mass and the mass of various WAT depots were substantially higher in old versus young animals (*Table 1*). 30-day calorie restriction reduced the body mass of young and old rats by 7.1% and 10.2%, respectively (*Table 1*). Subsequent *ad libitum* feeding restored the pre-CR body mass in already after 2 days in young rats, while the old rats fully regained their pre-CR body masses after 4 days of refeeding.

The total mass of the excised WAT depots accounted for 5.4% and 9.3% of the total body mass in young and old control rats, respectively, and was considerably higher in old animals (*Table 1*). The percentage of different WAT depots was similar in both age groups. CR decreased the total WAT mass by 37.7% and 24.2% in young and old rats, respectively. Although CR caused a reduction of fat mass in all three studied WAT depots, the relative decrease was highest in the perirenal WAT of young rats (–45.8%) and in the subcutaneous WAT of old animals (–26.6%) (*Table 1*). After 4 days of refeeding, the total WAT mass did not change in comparison to the CR group, in either young or old animals. A significant increase was noted only in the perirenal WAT of young rats (by 34.6% and 36.4% after 2 and 4 days of refeeding, respectively) (*Table 1*).

Effects of calorie restriction and refeeding on serum metabolite concentrations

Basal serum concentrations of triglycerides (TG) (*Fig. 1a*), total cholesterol, and HDL cholesterol (data not shown) were

Table 1. Changes in body mass and white adipose tissue mass of young and old rats subjected to short-term calorie restriction and refeeding.

	C	CR	CR+2	CR+4
YOUNG RATS				
Body mass [g]	347.9 ±4.55	322.5 ±3.03 ^{a}***	348.6 ±4.53 ^{b}***	355.9 ±11.71 ^{b}***
(pre-CR body mass)		(343.3)	(341.5)	(350.6)
Total sampled WAT [g]	18.75 ±1.26	11.68 ±0.61 ^{a}***	12.46 ±0.89 ^{a}***	13.54 ±0.67^{a}***
(% of body mass)	*(5.4%)*	*(3.6%)*	*(3.6%)*	*(3.8%)*
% of change vs. control		*37.7%*	*33.5%*	*27.8%*
pWAT [g]	5.28 ±0.44	2.86 ±0.19 ^{a}***	3.85 ±0.38 ^{a}*, ^{b}*	3.90 ±0.29 ^{a}*, ^{b}**
(% of tot. sampled WAT)	*(28.1%)*	*(24.5%)*	*(30.9%)*	*(28.9%)*
eWAT [g]	6.25 ±0.56	4.25 ±0.23 ^{a}**	4.33 ±0.35 ^{a}*	4.50 ±0.33 ^{a}*
(% of tot. sampled WAT)	*(33.3%)*	*(36.4%)*	*(34.8%)*	*(33.2%)*
sWAT [g]	7.23 ±0.49	4.57 ±0.34 ^{a}***	4.28 ±0.29 ^{a}***	5.13 ±0.29 ^{a}**, ^{c}*
(% of tot. sampled WAT)	*(38.6%)*	*(39.1%)*	*(34.3%)*	*(37.9%)*
OLD RATS				
Body mass [g]	519.6 ±7.78 ^{A}	469.5 ±7.28^{a}***,^{A}	479.5 ±12.25 ^{a}*,^{A}	498.0 ±13.28 ^{b}*,^{A}
(pre-CR body mass)		(527.1)	(525.0)	(529.2)
Total sampled WAT [g]	48.07 ±4.13^{A}	36.46 ±3.95^{a}*,^{A}	31.76 ±4.60^{a}*,^{A}	33.11 ±2.24^{a}*,^{A}
% of body mass	*(9.3%)*	*(7.8%)*	*(6.6%)*	*(6.7%)*
% change vs. control		*24.2%*	*34.0%*	*32.0%*
pWAT [g]	14.8 ±1.41^{A}	11.09 ±0.62^{a}*,^{A}	9.30 ±1.23^{a}*,^{A}	10.05 ±0.89^{a}*,^{A}
(% of tot. sampled WAT)	*(30.8%)*	*(30.4%)*	*(29.3%)*	*(30.3%)*
eWAT [g]	15.15 ±1.16 ^{A}	12.07 ±0.9^{a}*,^{A}	12.17 ±1.71 ^{A}	13.18 ±0.83^{A}
(% of tot. sampled WAT)	*(31.5%)*	*(33.1%)*	*(38.3%)*	*(39.8%)*
sWAT [g]	18.11 ±2.37 ^{A}	13.30 ±2.89^{a}*,^{A}	10.28 ±1.97^{a}**, ^{A}	9.88 ±0.71 ^{a}**,^{A}
(% of tot. sampled WAT)	*(37.7%)*	*(36.5%)*	*(32.4%)*	*(29.9%)*

Data represent means ±S.E.M. For individual WAT depots, the percentage of total sampled WAT mass is given. Small letters denote statistically significant differences vs. treatment group of either young or old rats (Mann-Whitney test): ^{a} - vs. control animals of the same age; ^{b} - vs. calorie-restricted rats; ^{c} - vs. calorie-restricted and re-fed for 2 days; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. The letter '^{A}' denotes statistically significant difference vs. corresponding data of young rats subjected to the same procedure (Mann-Whitney test), $p<0.05$. Symbols: **C** - control, *ad libitum* fed rats (young n=8, old n=8); **CR** - calorie-restricted (young n=10, old n=8); **CR+2** - calorie-restricted and re-fed for 2 days (young n=8, old n=8); **CR+4** - calorie-restricted and re-fed for 4 days (young n=8, old n=8); **pWAT**, **eWAT**, and **sWAT**: perirenal, epididymal, and subcutaneous white adipose tissue, respectively.

significantly higher in old rats, compared with young ones by 52%, 46% and 32%, respectively.

Short-term calorie restriction did not change total cholesterol or HDL cholesterol serum levels (data not shown), and decreased serum TG concentration (*Fig. 1a*) by 45% and 49% in young and old rats, respectively. Refeeding for 2 or 4 days did not significantly change TG, total cholesterol, and HDL cholesterol serum levels in either age group.

To assess the animals' nutritional statuses, albumin and total protein concentrations were measured in blood serum. Because no significant changes were noted for either age group (*Table 2*), it can be inferred that CR animals did not present gross malnutrition.

Effects of calorie restriction and refeeding on serum leptin concentration and depot-specific leptin gene expression

Serum leptin levels were threefold higher in control old vs. young rats (*Fig. 1b*). 30-day calorie restriction decreased serum leptin concentration by 84% and 50% in young and old rats, respectively. As compared with the CR animals, refeeding increased serum leptin concentration in young rats by 171% and 129% after 2 and 4 days, respectively, whereas no change occurred on refeeding in old rats. Leptin concentration in the control, *ad libitum* fed animals positively correlated with the mass of individual WAT depots, with the strongest correlation noted for the perirenal WAT, in both young (R^2=0.62, $p<0.001$) and old rats (R^2=0.70, $p<0.001$).

The relative contributions from each WAT site to leptin production were analysed by qPCR quantification of leptin gene expression (*Table 3*). Leptin mRNA levels did not change upon CR in any of the fat depots of young or old animals. Refeeding stimulated the gene expression in young rats' pWAT, but not in the other depots. In old rats such an induction was noted first in eWAT (3.5-fold increase vs. control after 2-day refeeding), and later in pWAT (2.5-fold increase after 4-day refeeding). Thus, in terms of leptin gene expression, the perirenal depot appeared to

Table 2. Blood serum concentrations of albumins and total protein in young and old rats subjected to short-term calorie restriction and refeeding.

	C	CR	CR+2	CR+4
		YOUNG RATS		
Serum albumin [g/L]	13.63 ±0.21	13.90 ±0.30	13.25 ±0.55	13.00 ±0.25
Serum protein [g/L]	58.24 ±0.46	56.33 ±0.70	54.71 ±0.94	54.38 ±0.39
		OLD RATS		
Serum albumin [g/L]	12.00 ±0.28 ***	12.50 ±0.40 *	13.00 ±0.53	12.38 ±0.28
Serum protein [g/L]	61.00 ±0.52	60.13 ±0.93 *	56.50 ±1.17	61.14 ±1.02

Data represent means ±S.E.M. Statistically significant differences vs. young animals (Mann-Whitney test) are denoted by: * $p<0.05$; *** $p<0.001$. No differences were observed between experimental groups of animals subjected to different dietary regimens. Symbols and number of animals per group as in *Table 1*.

Table 3. Leptin gene expression in white adipose tissue depots of young and old rats subjected to short-term calorie restriction and refeeding.

	C	CR	CR+2	CR+4
		YOUNG RATS		
pWAT	1 ±0.25	0.73 ±0.25 ^{a*,b}**	2.89 ±0.67	1.16 ±0.24
eWAT (relative to pWAT)	1 ±0.35 (1.47)	0.94 ±0.38	0.62 ±0.40	0.90 ±0.45
sWAT (relative to pWAT)	1 ±0.21 (0.74)	0.32 ±0.1	0.96 ±0.38	0.99 ±0.39
		OLD RATS		
pWAT	3.45 ±1.50	1.81 ±1.05	3 ±1.45	8.45 ±1.19 ^{b*,c*,A}**
eWAT (relative to pWAT)	1.96 ±0.88 (0.83)	3.17 ±1.31	7.35 ±2.77 ^{a*,A}*	5.87 ±1.45 ^{a*,A}*
sWAT (relative to pWAT)	0.73 ±0.24 (0.17)	0.58 ±0.31	0.85 ±0.44	1.06 ±1.36

Data represent leptin gene expression in experimental groups (means ±S.E.M.) relative to the mean values obtained for young control rats (expression level=1) in each WAT depot studied. For the control groups, fold change between the expression in eWAT and sWAT relative to pWAT (expression level =1) is given in brackets. Indications of statistically significant differences, symbols, and number of animals in groups, as in *Table 1*.

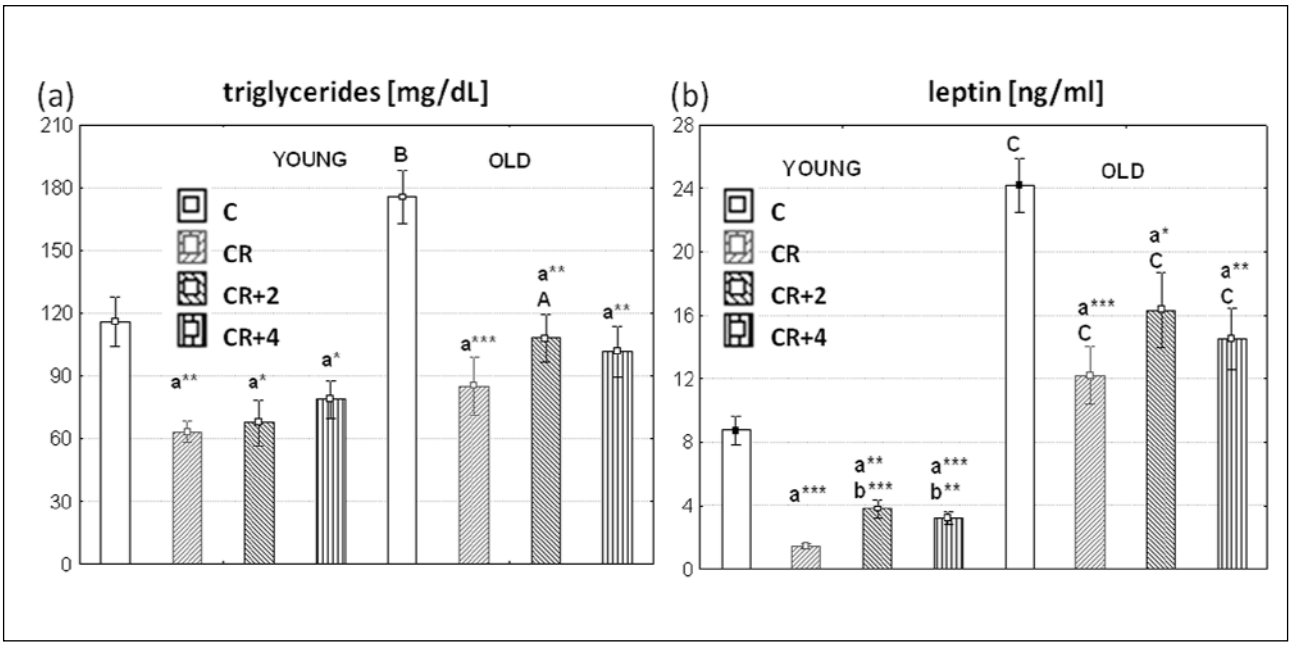

Fig. 1. Effects of calorie restriction and refeeding on serum concentration of triglycerides (a) and leptin (b) in young and old rats. Data represent means ± S.E.M. Symbols and number of animals in groups are as in *Table 1*. Small letters denote statistically significant differences vs. treatment group of either young or old rats (Mann-Whitney test): [a] - vs. control animals of the same age; [b] - vs. CR rats; [c] - vs. CR+2 rats (*i.e.* rats for 2 days after SCR); * p<0.05; ** p<0.01; *** p<0.001. Capital letters denote statistically significant differences vs. corresponding data of young rats subjected to the same procedure (Mann-Whitney test), [A], p<0.05; [B], p<0.01; [C], p<0.001.

Table 4. WAT depot-specific changes in lipogenic enzymes' activities in response to short-term calorie restriction and refeeding - summary of obtained results.

		C	*p vs.* old	CR	*p vs. C*	CR+2	*p vs. C (CR)*	CR+4	*p vs. C (CR)*
YOUNG RATS									
pWAT	**ACL**	5.48	<0.001	↑	<0.01	↑	<0.01	↑	<0.01
	FAS	1.72	<0.01	=	NS	↑	<0.01 (<0.01)	↑	<0.01 (<0.05)
	G6PD	24.54	NS	↑	<0.001	↑	<0.001(<0.01)	↑	<0.001 (<0.05)
eWAT	**ACL**	4.12	<0.001	=	NS	=	NS	↑	<0.01
	FAS	1.63	<0.01	=	NS	↑	NS (<0.05)	=	NS
	G6PD	17.05	<0.05	↑	<0.01	↑	<0.05	=	NS
sWAT	**ACL**	3.11	<0.01	=	NS	↑	<0.01 (<0.01)	↑	<0.01 (<0.01)
	FAS	3.39	<0.05	=	NS	=	NS (<0.001)	↑	<0.01 (<0.001)
	G6PD	18.64	NS	=	NS	=	NS	=	NS
OLD RATS									
pWAT	**ACL**	1.54		=	NS	↑	<0.05	↑	<0.05 (<0.05)
	FAS	0.55		↓	<0.01	=	NS (<0.01)	=	NS (<0.01)
	G6PD	22.14		=	NS	=	NS	=	NS
eWAT	**ACL**	0.85		=	NS	=	NS	=	NS
	FAS	0.30		=	NS	=	NS	=	NS
	G6PD	12.15		=	NS	↑	<0.05 (<0.05)	=	NS
sWAT	**ACL**	1.43		↓	<0.05	=	NS	=	NS (<0.01)
	FAS	1.24		↓	<0.01	=	NS (<0.05)	=	NS (<0.05)
	G6PD	20.67		=	NS	↓	<0.05 (<0.05)	=	NS

The numerical values given for control groups correspond to enzymatic activity expressed in nmol/min/mg protein. For calorie-restricted and groups, changes in enzymatic activity vs. control animals are indicated by ↑ (increase), ↓ (decrease), and = (no change). Statistically significant differences vs. control and vs. calorie-restricted animals (in brackets) are shown; NS - not significant. ACL, ATP-citrate lyase; FAS, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase. Other symbols, and number of animals in groups, as in *Table 1*.

be most responsive in young rats, alongside epididymal WAT in old rats. Although leptin mRNA levels tended to be the highest in eWAT of the young and pWAT of the old animals, the differences between various depots in a given age group were not statistically significant.

Effects of calorie restriction on the activities of lipogenic enzymes in various white adipose tissue depots

Changes of lipogenic enzymes' activities in young and old rats subjected to the experimental procedures are presented in *Fig. 2* and summarized in *Table 4*.

ATP-citrate lyase

Basal ATP-citrate lyase (ACL) activity was 2–4 times lower in the studied WAT depots of control old rats as compared to the young ones (*Fig. 2a*). Activity was similar in all the studied WAT depots in the control young rats. In old rats, however, ACL activity was the lowest in the epididymal WAT and similar in the other depots (*Fig. 2a*).

In young rats, 30-day calorie restriction resulted in a strong, sevenfold induction of ACL activity in the perirenal WAT, with a trend towards further increases after 2 days of refeeding (by 83% versus CR group, p=0.056). Activity remained elevated after 4 days of refeeding (*Fig. 2a*). In contrast to young rats, calorie restriction did not change ACL activity in the pWAT of old rats, whereas refeeding for 2 or 4 days significantly increased the enzyme activity by 79% and 68%, respectively (*Fig. 2a*).

In the epididymal WAT (eWAT), ACL activity was fairly unresponsive to dietary manipulations in both young and old rats, with the exception of increased ACL activity in the eWAT of young rats re-fed for 4 days (*Fig. 2a*).

In the subcutaneous WAT (sWAT) of young rats, ACL activity did not change after calorie restriction. It was, however, stimulated fourfold following 2 and 4 days of refeeding (*Fig. 2a*). In contrast to young rats, CR caused a twofold decrease of ACL activity in the sWAT of old animals, which reverted to the control level upon refeeding.

Fatty acid synthase

In all the studied WAT depots, fatty-acid synthase (FAS) activity was 3–4 times lower in old than in young *ad libitum* fed control rats (*Fig. 2b*). The activity of this enzyme appeared to be higher in the subcutaneous WAT, as compared with the two other WAT locations, in both age groups, although the differences did not reach statistical significance (*Fig. 2b*).

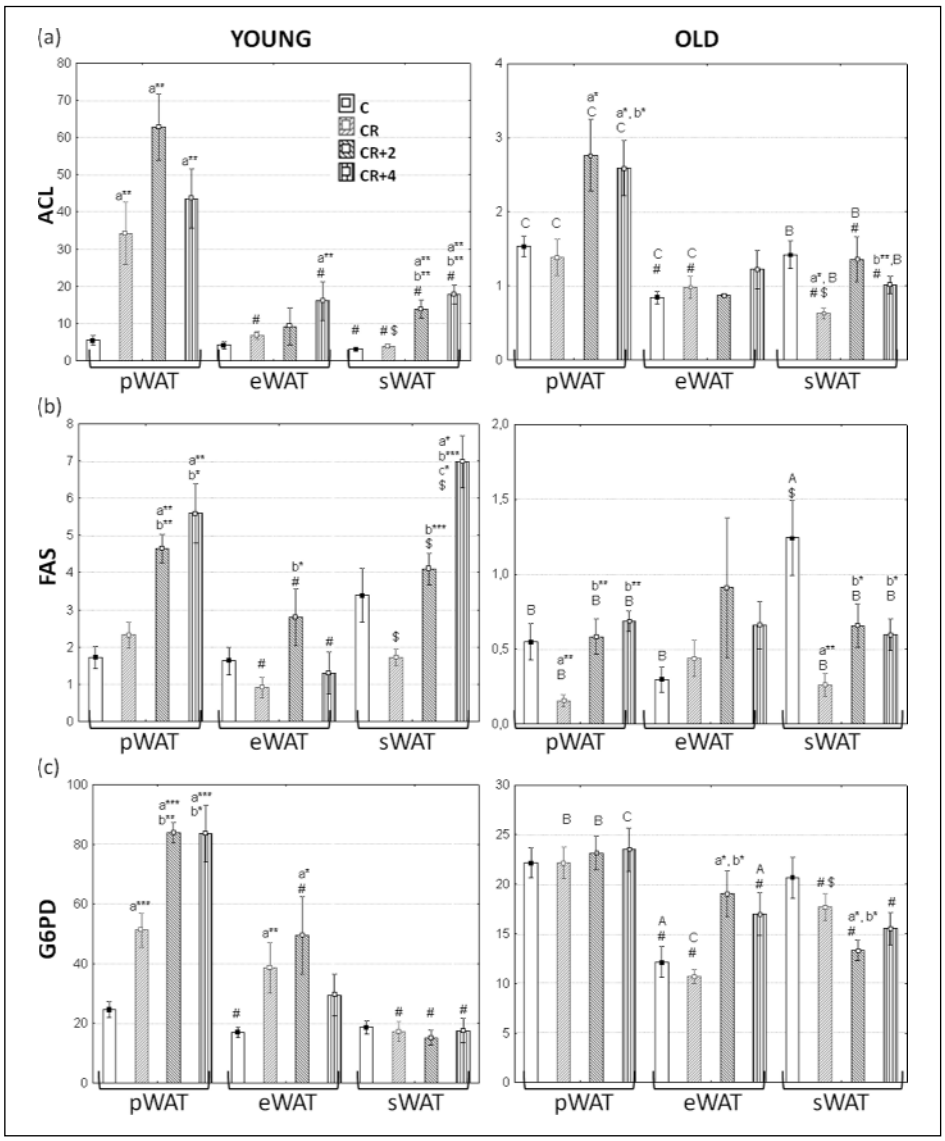

Fig. 2. Activities of lipogenic enzymes in WAT depots of young and old rats subjected to calorie restriction and refeeding. Enzymatic activities of (**a**) ACL, (**b**) FAS, and (**c**) G6PD, measured as described in Methods, are expressed as the amount of NADH oxidized (ACL), NADPH oxidized (FAS), and NADP reduced (G6PD) per minute per mg tissue protein. For clarity, different ranges of activity units are shown on the Y axes for young and old rats. Data represent means ± S.E.M. Statistically significant differences between various WAT depots (Wilcoxon signed-rank test) are denoted by: $^{\#}$, vs. pWAT ($p<0.05$); and $^{\$}$, vs. eWAT ($p<0.05$). Other symbols and number of animals in groups are as in *Table 1*.

Calorie restriction did not affect FAS activity in the perirenal WAT of young rats, while refeeding resulted in a 2.5-fold induction of its activity (*Fig. 2b*). On the contrary, in the pWAT of old rats, CR led to a threefold decrease of FAS activity, while refeeding restored the enzyme's basal activity level (*Fig. 2b*).

In the epididymal WAT of young and old rats, calorie restriction did not alter FAS activity (*Fig. 2b*). Refeeding for 2 days led to a threefold increase in FAS activity in young rats, whereas after refeeding for 4 day, the activity returned to basal level. In old rats, however, refeeding did not change FAS activity in the epididymal WAT (*Fig. 2b*).

In the subcutaneous WAT of young rats, FAS activity tended to decrease upon CR (p=0.058), whereas refeeding for 2 and 4 days increased the enzyme's activity by 110% and 270% above the CR level, respectively (*Fig. 2b*). In old rats, calorie restriction resulted in an almost fourfold decrease in FAS activity, while refeeding for 2 and 4 days increased FAS activity versus CR animals by 154% and 131%, respectively. However, the activity was still 50% lower than in control old animals.

Glucose-6-phosphate dehydrogenase

In control young and old rats, glucose-6-phosphate dehydrogenase (G6PD) activity was at similar levels in the perirenal and the subcutaneous WAT depots, whereas in the epididymal WAT it was lower by 29% in old as compared with young animals. In both age groups, G6PD activity was approximately 40% lower in eWAT than in pWAT (*Fig. 2c*).

In the perirenal WAT of young rats, 30-day calorie restriction resulted in a twofold increase in G6PD activity, and refeeding led to a further increase (fourfold versus control). On the contrary, neither CR nor refeeding induced any changes in G6PD activity in the pWAT of old rats (*Fig. 2c*).

In the epididymal WAT of young animals, as in the perirenal WAT, CR induced a twofold increase in G6PD activity, which was sustained after 2 and 4 days of refeeding (*Fig. 2c*). In old animals, unlike in young rats, G6PD activity did not change upon CR, but it did increase following 2 days of refeeding, and remained slightly elevated after 4 days (*Fig. 2c*).

Regardless of dietary manipulation, G6PD activity levels were similar in the subcutaneous WAT of young rats. In old rats, short-term calorie restriction did not affect the activity of the enzyme, but after refeeding for 2 days G6PD activity decreased (by 33% and 25% versus control and CR, respectively) and returned to basal level after 4 days of refeeding (*Fig. 2c*).

DISCUSSION

During aging, decreased lipogenic capacity and lower activity of lipogenic enzymes in white adipose tissue (27) are accompanied by the accumulation and redistribution of fat tissue in humans and rodents (5, 6). Although in rodents and non-human primates, long-term or lifelong calorie restriction (LCR) promotes health and delays age-related diseases (28), this type of dietary manipulation cannot be successfully implemented in humans for psychological and ethical reasons. Short-term calorie restriction (SCR) regimes have been introduced to decrease the body mass of overweight humans, including the elderly, but their effects on metabolic homeostasis have not been thoroughly characterized (29, 30). The efficacy of calorie-restricted regimes is hampered by the regaining of body mass, mainly fat mass, after the reintroduction of the maintenance diet ('rebound effect'). In the current study we attempted to explore the effects of SCR, refeeding, and aging on some parameters of lipid homeostasis three major WAT depots and at systemic level in rats.

Our current study is the first one to systematically compare serum concentrations of leptin and several metabolites and the activities of lipogenic enzymes in three major WAT depots of young and old rats in *ad libitum* fed and short-term dietary-restricted animals. We showed that SCR and refeeding differently affected the lipogenic function of WAT in rats, depending on the depot localization, the age of the animals, and the type of enzyme. For this purpose we used male Wistar rats, as the alterations in their body mass over the lifespan mimic the situation in humans: body mass in this strain reaches its peak in middle age or early old age, and often decreases in the final phase of life (1). Previous studies showed that body weight and adiposity in rat gradually increase starting already from 12 weeks of age (31).

Similarly, we also found an age-related increase in the total WAT mass, the percentage of which in relation to body mass almost doubled between the age of 5 and 24 months. Interestingly, in contrast to human studies that have documented the redistribution of fat stores in the elderly (4, 6), aging in the studied rat strain did not alter the relative proportions of the major regional fat depots. Similarly to Sucajtys-Szulc *et al.* (20), we found that, in young rats, the perirenal (retroperitoneal) adipose tissue (pWAT), as compared to the epididymal and subcutaneous WAT depots, showed the highest responsiveness to 30-day dietary restriction and subsequent refeeding, manifested by highest stimulation of lipogenic enzymes' activity. Interestingly, after refeeding, the total sampled WAT mass did not reach the control level in either age group, and was about 30% lower - a finding previously reported for young rats (20) and mice (32).

The elevated serum concentrations of triglycerides and total cholesterol - a common feature of aging in rodents and humans (14, 33, 34) - may contribute to an increased risk of atherosclerosis and cardiovascular diseases (35). We, like Kim *et al.* (14), have found that short-term calorie restriction decreased serum TG concentration in both young and old rats. This effect was sustained throughout 4 days of refeeding, despite the strong induction of the activity of hepatic lipogenic enzymes (data not shown). These findings indirectly correspond to those of Kochan *et al.* (36), who found decreased plasma TG concentration after refeeding for 9 days in young rats subjected to multiple cycles of starvation and refeeding. Thus, short-term calorie restriction decreased serum TG concentration in rats, regardless of age, and this effect sustained during first days of refeeding.

One well-established effect of long-term calorie restriction is the increased sensitivity of adipose tissue to lipogenic stimuli, which encompasses higher CR-induced expression and activity of lipogenic enzymes, expression of lipogenic/adipogenic transcription factors, and density of insulin receptors (37-39). However, the data concerning the effects of short-term calorie restriction in lipogenic tissues are inconclusive, and no attempts so far have been made to discern the role of different WAT depots in these outcomes.

Perirenal WAT was generally more sensitive to calorie restriction and refeeding than were epididymal or subcutaneous WAT, and this was reflected in the respective alterations of ACL and G6PD activities. However, FAS activity did not change after 30 days of CR in the perirenal WAT of young rats, possibly because too short a CR period was applied. Accordingly, Mulligan *et al.* (40) found in mice that mRNA levels of FAS and its main regulator, SREBP-1c, increased in the epididymal and subcutaneous WAT only after 16 weeks (and not after 1 or 4 weeks) of calorie restriction. Moreover, the study of Stelmanska *et al.* (41) demonstrated that the induction of malic enzyme activity in the epididymal WAT of young rats depended on the duration and severity of calorie deprivation, with higher induction of activity in rats subjected to 50% CR than in those subjected to 30 days of 85% CR.

The epididymal WAT depot functionally resembles omental fat in humans (6). In rats, we found significant functional differences between the two visceral fat depots, pWAT and eWAT. The epididymal WAT appeared to be remarkably more inert under changing nutritional conditions: in young rats, no induction of ACL or FAS activity was found following 30 days of CR, while the induction of enzymes by refeeding was of much smaller magnitude than in the perirenal WAT. In concert with the higher replicative capacity of perirenal preadipocytes over epididymal preadipocytes in rats (6), these differences in the lipogenic potential of regional tissue depots may suggest a major role for perirenal, as opposed to epididymal, fat in the regaining of weight shortly after the cessation of calorie restriction in humans. Similarly to our data on enzymatic activities, no differences were found in *Fasn* transcript levels between epididymal and subcutaneous WAT in young male mice subjected to 4 weeks of 60% calorie restriction (40). On the contrary, *Fasn* induction was demonstrated in the epididymal adipose tissue of Fischer 344 rats kept on 60% diet for either 2 or 25 consecutive months after weaning (39). The discrepancies between the studies may be related to strain type, duration, and time of CR initiation, as well as the different analytical methods applied.

In the subcutaneous WAT, the response to SCR and refeeding was substantially different in young and old rats, with ACL and FAS activities unaffected by CR in young animals, but significantly reduced in old ones, and stimulated above basal level upon *ad libitum* feeding of young, but not old, animals. In concert with the reduced enzymatic activity, the subcutaneous WAT of old rats presented the greatest decline in tissue mass upon dieting. These observations suggest a reduced fat storage capacity in the subcutaneous adipocytes of old rats. The limited response to refeeding of subcutaneous WAT in comparison to the perirenal WAT confirms previously published data, which showed that subcutaneous WAT was characterized by lower triglyceride turnover (42). On the other hand, different conditions (*i.e.* prolonged statin administration) resulted in weight gain in subcutaneous but not perirenal and epididymal WAT depots (43). These discrepancies may be explained by activation of diverse signaling mechanisms by different obesogenic factors.

The heterogeneous outcome of dieting in different fat pads is unlikely to be attributable to differences in WAT depot-specific secretory activity. Even though subcutaneous adipose tissue tended to release less pro-inflammatory cytokines, no overt differences in secretome were noted between inguinal and epididymal fat of normally fed mice (44). Moreover, no differences were observed between the CR-induced changes in cytokines' release from retroperitoneal and mesenteric tissues (45).

A report by Bruss *et al.* (46) demonstrated that CR causes cyclic changes in lipid metabolism in adipose tissue, with alternating periods of increased lipogenesis and enhanced fatty acid oxidation. Therefore, CR diets may decrease WAT mass due to higher lipid oxidation over synthesis. Although we did not perform a time-course of changes of the lipogenic enzymes' activities during 30 days of calorie restriction, the possibility of intermittent cycling in lipid metabolism during CR (46) could explain the induction of enzymatic activities upon refeeding observed in our model. Thus, the reprogramming of lipid metabolism regulation by CR in adipocytes would lead to preferential lipid deposition when nutrient intake increases during refeeding period. Analysis of the depot-specific differences in response to short-term CR and refeeding suggests that perirenal WAT is the major site 'activated' by CR, particularly in young animals. Our findings stay in contrast to those reported by Bruss *et al.* (46), who pointed to subcutaneous adipose tissue as the primary site for endogenous fatty acid synthesis in young calorie-restricted mice. A second mechanism responsible for the 'rebound effect' of refeeding could involve hyperphagia. Re-fed rodents have been reported to increase their *ad libitum* food consumption, which led to hyperinsulinemia and enhanced anabolism (47).

Similarly to previous reports (17, 27, 39), we observed a general age-related decline in the lipogenic capacity of adipose tissue, as illustrated by the decreased ACL and FAS activities in all the studied WAT depots. G6PD activity, which provides NADPH for the synthesis of fatty acids, was generally unaffected by old age. Despite the decreased activity of FAS and ACL, old rats had markedly higher adipose tissue mass than young rats. This phenomenon may be explained by reduced utilization of lipids, as the rate of fatty acid oxidation diminishes during aging (48). Moreover, old animals in our study consumed more food than young ones.

Similarly to previous reports (31, 33, 49), we confirmed that old rats had markedly elevated serum leptin concentration. Because leptin inhibits FAS activity, increased leptin concentration in old rats may contribute to the age-related decrease in WAT lipogenic activity (49, 50). The adaptive capacity of WAT to rapid changes of nutritional environment seems to be suppressed in aging: serum concentration of leptin did not change upon refeeding of old rats following 30 days of calorie restriction, in contrast to young animals, in which it almost doubled after 2 and 4 days of refeeding. Similar unresponsiveness of WAT to refeeding in respect to serum leptin level has also been reported by Kmiec *et al.* (32) in old rats subjected to 2 days of starvation. The decreased serum levels of leptin in rats subjected to short-term CR may be related to lower WAT mass or post-transcriptional regulation, though not decreased leptin gene expression, which did not change upon dieting in any of the depots studied. In contrast, Sucajtys-Szulc *et al.* (20) have shown lower abundances of *Lep* mRNA in the perirenal WAT of young rats fed a 50% calorie restricted diet for 30 days. In humans, six months of 25% calorie restriction resulted in a 44% decrease in mean 24-hour circulating leptin concentrations, but no data were reported on leptin levels after the return to normal diet (51). Our study revealed that the refeeding-associated increase in serum leptin concentration may result from the induction of the *Lep* gene expression in perirenal WAT. Moreover, this response appears to be delayed in old animals, which might explain why there was no significant increase of serum leptin level within the four days of refeeding in our experimental setting.

The CR model adapted for this study was based on a non-supplemented commercial diet restricted to 60% of the *ad libitum* food intake. Though it has been previously reported that such a dietary regimen may lead to micronutrient malnutrition, supplementation appears to be crucial particularly for young animals (52). Rodents who received vitamin and mineral supplements with a long-term CR diet gained significantly more weight than their equicaloric, non-supplemented counterparts (52). In the present study, the animals did not present gross malnutrition, as suggested by lack of alterations in serum albumin and total protein levels. In humans, very low calorie diets, with comparable limitation of calorie intake, are implemented for the purpose of quick weight loss; however, vitamin and micronutrient content of such diets are strictly regulated (8).

In humans, in parallel to our rodent model, we anticipate moderate weight regain after dieting in the elderly as compared to adults, and relatively highest contribution of the perirenal fat to this rebound effect. However, we cannot repudiate the influence of diet composition on these results.

In summary, we systematically compared the activities of lipogenic enzymes (FAS, ACL and G6PD) in three major white adipose tissue depots of young and old rats subjected to 30 days of a calorie-restriction regime and subsequent refeeding for 2 and 4 days. We found that WAT localization affected the pattern of the lipogenic enzymes' response to SCR and refeeding. Because the

perirenal WAT showed the highest activity of lipogenic enzymes both in basal conditions and after refeeding in young and old rats, this depot may be predominantly responsible for weight regain in calorie-restricted subjects after switching back to normal diet. The results of our investigation should instigate further studies in humans on the effectiveness of SCR as a means to decrease body mass in older people. If our findings on age-related decrease in the rate of lipogenic enzymes' induction upon refeeding extend to humans, short-term calorie restriction might prove to be more effective for body-mass reduction in old than in young people due to reduced 'rebound effect'.

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