Age-Related Changes in Sirtuin 7 Expression in Calorie-Restricted and Refed Rats

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Abstract
Background: Sirtuins (SIRT1–7) have been implicated to mediate the beneficial effects of calorie restriction for healthy aging. While the physiological functions of SIRT7 are still poorly understood, SIRT7 has recently been shown to affect ribosome biogenesis, mitochondrial gene expression, and hepatic lipid metabolism. Objective: To analyze the effects of age and short-term calorie restriction (SCR) and subsequent refeeding on SIRT7 expression in key metabolic tissues. Methods: Four- and 24-month-old male Wistar rats were subjected to 40% SCR for 30 days, followed by ad libitum feeding for 2 or 4 days. Liver, white adipose tissue (WAT), heart and skeletal muscle samples were analyzed by real-time PCR and Western blotting for SIRT7 mRNA and protein expression, respectively. Results: Aging had diverse effects on SIRT7 levels in lipogenic tissues: both the mRNA and protein levels increased in the retroperitoneal depot (rWAT), did not change in the epididymal depot (eWAT), and decreased in the subcutaneous depot (sWAT) and the liver of old as compared to young animals. In the heart, extensor digitorum longus muscle (EDL) and soleus muscle (SOL), Sirt7 gene but not protein expression was lower in old than in young control rats. SCR did not affect SIRT7 expression in WAT and the liver in both age groups. In the heart of young animals, SCR did not affect SIRT7 mRNA or protein level. In EDL, SIRT7 protein but not mRNA levels decreased after SCR and remained reduced upon refeeding. In SOL, both SIRT7 mRNA and protein expression were inhibited by refeeding. In old rats, cardiac Sirt7 expression increased after SCR and refeeding. In old rats’ EDL and SOL muscles, SIRT7 protein expression was inhibited by refeeding. Conclusion: Age-related changes of SIRT7 gene expression in key organs of energy homeostasis are tissue dependent.

Introduction
Sirtuins (SIRT1–7) are a family of proteins that in mammals regulate key cellular processes, including response to stress, energy metabolism, cell cycle, differentiation, and apoptosis [1]. Earlier studies suggested a crucial role of sirtuins in promoting longevity [2], although only the overexpression of SIRT1 [3] and SIRT6 [4] was A.W. and A.L. contributed equally to this work.
demonstrated to extend the lifespan in mice, while in men a polymorphism of SIRT3 was shown to correlate with prolonged lifespan [5]. Nevertheless, numerous studies on sirtuin knockout animal models underscore the importance of sirtuins for healthy aging.

SIRT7 involvement in aging and age-associated pathologies was first observed in Sirt7 knockout mice, which showed markedly reduced mean and maximum lifespans, heart hypertrophy, inflammatory cardiomyopathy, and extensive cardiac fibrosis [6]. Moreover, recent studies suggest that SIRT7 may be involved in the regulation of key metabolic processes [7–10]. For example, the Sirt7−/− phenotype was characterized by fatty liver [8, 9] which was reverted by liver-specific SIRT7 overexpression, while in normal mice fed high-fat diet overexpression of SIRT7 prevented hepatic steatosis [9]. The role of SIRT7 in aging was further supported by finding its reduced expression in aged hematopoietic stem cells (HSCs), while SIRT7 upregulation enhanced the regenerative capacity of aged hematopoietic stem cells (HSCs) [9]. Moreover, the role of SIRT7 as a protective molecule in conditions of cellular stress such as unfolded protein response has also been suggested recently [10].

Sirtuins are NAD+/NADH ratio dependent deacetylases; thus, their enzymatic activity can be modulated by the cellular NAD+/NADH ratio [1]. The redox status in the cell changes under conditions of low nutrient supply such as calorie restriction, which increases NAD+ availability in muscle and white adipose tissue (WAT) and may thus stimulate sirtuin activity [11]. Indeed, restricted diet was shown to alter SIRT1, SIRT3, and SIRT6 expression in murine and rat tissues [12–14]. Interestingly, there are no data on the effects of this nutritional manipulation on SIRT7 expression. In our previous study, we have shown that short-term calorie restriction (SCR) and subsequent refeeding differentially altered the activities of lipogenic enzymes in WAT depots [15]. Due to the increasing evidence on SIRT7’s role in the regulation of mitochondrial function [8] and key metabolic processes involved in energy homeostasis [9], we decided to assess Sirt7 gene expression at the mRNA and protein levels in key tissues involved in energy metabolism in young and old male rats subjected to SCR and refeeding. We analyzed SIRT7 expression in the liver, three major WAT depots (retroperitoneal, rWAT; epididymal, eWAT; subcutaneous, sWAT), heart muscle, and skeletal muscles: soleus (SOL; composed predominantly of red fibers) and extensor digitorum longus (EDL; predominantly white fibers).

### Material and Methods

#### Animals and Tissue Collection

The experiment was performed on young-adult (4-month-old at the onset of the experiment) and old (24-month-old) male Wistar-Han rats maintained under specific pathogen-free conditions in the Academic Animal Experimental Centre in Gdansk, Poland, at 22 °C under a 12-hour light/12-hour dark cycle. Littermates were housed in cages individually or in pairs of similar weight. All animals were fed a standard, sterilized Chow (Labofeed H; Pasze Morawski, Kcynia, Poland; detailed composition in Turyn et al. [16]), and had free access to water. Food intake was determined during a 3-week-long pre-trial period, after which rats were randomly assigned to SCR (n = 26/24, young/old animals, respectively) or control, ad libitum fed group (n = 10/10). Animals of the SCR group received 60% of the baseline food intake for 30 days on a daily basis, 2 h after lights on. At the end of the dieting period, some animals were sacrificed (n = 10/8), while others were refed ad libitum for 2 days (n = 8/8) or 4 days (n = 8/8). The animals were euthanized under full anesthesia (ketamine 90 mg/kg and xylazine 10 mg/kg i.p.), which was followed by blood acquisition by heart puncture, and quick tissue collection. Tissue samples of the cardiac muscle from the heart’s apex, liver, SOL and EDL muscles, and three depots of WAT (rWAT, eWAT, and sWAT) were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. All animal experimental procedures had been authorized by the local ethics committee and were conducted in agreement with the institutional guidelines of the Medical University of Gdansk, Poland.

#### Gene Expression

Gene expression was analyzed by two-step real-time PCR as described in Wronska et al. [15]. Sirt7 mRNA was quantified relative to the expression of acidic ribosomal phosphoprotein P0 (36B4) and cyclophilin A (CycloA) in the heart and skeletal muscles, and relative to 36B4 and β-actin in the liver and WAT samples. The primers were designed using Primer3Plus software based on BLAST, Ensembl, and AceView databases. The primer pairs were: Sirt7 5′-TGAATGTGATGTAATGCGGCTCCTC-3′ and 5′-CTGCTCCTGGC- CACATGTACTTCC-3′, 36B4 5′-CTCAGTGCTCCTACTCAT-CA-3′ and 5′-GGGGCTTAGTCGAAGAGACC-3′, CycloA 5′-TG- TCTCTTTTTCGCCGCTTGCTG-3′ and 5′-CACACCCTGCGCC- ACAATGAAATCC-3′, and 36B4 5′-GAAATCGTGCGTGACATTA- G-3′ and 5′-GCTAGAACATTTGCCGTTGA-3′.

#### Western Blotting

Measurements of SIRT7 protein level were performed in triplicate for young and old rats from the control, SCR, and 4-day-long refeeding groups. Tissue lysates were prepared with the Mammalian Cell Extraction Kit (BioVision, Milpitas, Calif., USA). Protein concentration was measured using Bradford reagent (Sigma-Al- drich, Saint Louis, Mo., USA); 20-µg protein samples were separated on 10% SDS-PAGE gels, transferred to PVDF membranes (Invitrogen – Life Technologies, Carlsbad, Calif., USA), and blocked with 5% nonfat milk in TBS. The membranes were incubated overnight at 4 °C with anti-SIRT7 antibodies (bs-5973R, Bioss, Woburn, Mass., USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (A9046, Sigma-Aldrich) for 2 h at room temperature. Bands were visualized using Chemiluminescent Peroxidase Substrate (Sigma-Aldrich) and developed against light-sensitive X-ray.
**Fig. 1.** SIRT7 gene (a) and protein (b) expression in lipogenic tissues of young and old rats subjected to SCR and refeeding. **a** Sirt7 mRNA levels in the liver, rWAT, eWAT, and sWAT were measured by real-time PCR and normalized to young control animals (expression level = 1). Data represent means ± SEM of young (light grey bars) and old (dark grey bars) rats, 8–10 animals per group. Significant differences: a, vs. control animals of the same age; b, vs. calorie-restricted rats; c, vs. rats refed for 2 days (Kruskal-Wallis ANOVA); *p < 0.05, significant difference between corresponding groups of young and old rats (Mann-Whitney U test). **b** SIRT7 protein levels in the control, calorie-restricted, and 4-day-long refed rats were assessed by Western blotting, with GAPDH as the loading control. Representative results of three measurements are shown. C = Control animals; SCR+2 and SCR+4 = animals calorie-restricted and refed for 2 and 4 days, respectively.
films. SIRT7 protein levels were analyzed relative to GAPDH levels (monoclonal horseradish peroxidase-conjugated antibodies; G9295, Sigma-Aldrich) using QuantityOne software (Bio-Rad, Hercules, Calif., USA).

Statistical Analysis

Because not all sets of data followed normal distribution (Shapiro-Wilk test), statistical analyses were performed using the non-parametric Kruskal-Wallis ANOVA for multiple comparisons and the Mann-Whitney U test for paired analysis. Statistical significance was accepted at \( p < 0.05 \). Calculations were performed with Statistica v.10 software (StatSoft, Cracow, Poland).

Results

Effects of Aging on SIRT7 Gene and Protein Expression of Normally Fed Rats

Under normal feeding conditions Sirt7 gene expression varied in different lipogenic tissues depending on animals’ age. Sirt7 mRNA level was 2-fold lower in the liver of old than young control rats (fig. 1a). In various WAT depots of old rats, Sirt7 expression was 30% lower in sWAT, 3-fold higher in rWAT, and similar in eWAT as compared with young rats (fig. 1a). SIRT7 protein level decreased in the liver and in sWAT, increased in rWAT, and did not change in eWAT in the old as compared to young rats’ tissues (fig. 1b).

In old as compared to young control rats, Sirt7 mRNA levels decreased more than 2-fold in the heart, EDL, and SOL muscles (fig. 2a). However, no marked reductions in SIRT7 protein levels were observed in these tissues (fig. 2b).

Effects of SCR and Refeeding on SIRT7 Gene and Protein Expression in Lipogenic Tissues

Neither SCR nor subsequent ad libitum feeding caused any change in Sirt7 gene expression in lipogenic tissues of
young rats (fig. 1a). In old rats, SCR alone did not affect Sirt7 gene expression in either the liver or WAT depots. However, refeeding for 2 days decreased Sirt7 mRNA levels in the liver (2-fold) and rWAT (2.5-fold), which returned to control values after 4 days of ad libitum feeding. Sirt7 expression was not affected by SCR or refeeding in eWAT and sWAT (fig. 1a). SIRT7 protein expression in the liver and WAT did not change in response to SCR or subsequent 4-day-long refeeding in either young or old rats (fig. 1b).

**Effects of SCR and Refeeding on SIRT7 Gene and Protein Expression in Cardiac and Skeletal Muscles**

In young rats, SCR and refeeding did not affect SIRT7 gene or protein expression in the cardiac muscle (fig. 2). Neither SCR nor refeeding affected Sirt7 mRNA levels in the EDL muscle of young rats (fig. 2a). However, SCR decreased SIRT7 protein expression, which remained below control level after the full-diet feeding (fig. 2b). In the SOL muscle, SIRT7 gene and protein expression did not change upon SCR. Subsequent ad libitum feeding decreased both the mRNA and SIRT7 protein levels as compared to SCR rats (fig. 2).

In old rats, SCR caused a 2-fold increase in Sirt7 expression in the heart muscle, with the elevated mRNA levels persisting throughout the refeeding period (fig. 2a). However, there was no increase in cardiac SIRT7 protein level (fig. 2b). In EDL of old animals, SCR increased Sirt7 expression by 95%; the elevated mRNA level was maintained for 2 days of refeeding and decreased to control values thereafter (fig. 2a). SIRT7 protein expression in EDL did not change after SCR but decreased after a 4-day-long refeeding (fig. 2b). In the SOL muscle of old rats, SCR and refeeding had no effect on Sirt7 gene expression (fig. 2a). SIRT7 protein expression in SOL did not change during SCR; however, it decreased upon refeeding (fig. 2b).

**Discussion**

We present a comprehensive analysis of age-related and tissue-specific expression of SIRT7 in key rat tissues involved in fuel and energy metabolism. Moreover, we show that dietary manipulations such as SCR and subsequent refeeding differently affect SIRT7 mRNA and protein levels in the cardiac and skeletal muscles of young and old rats.

Our study provides novel data on the age-related SIRT7 expression in three major depots of WAT. These findings provide yet more proof of WAT’s metabolic diversity [17]. Such divergent age-related changes may reflect different roles of SIRT7 in various fat depots. Although nervous tissue completely differs in morphology and function from WAT, it is worth noting that SIRT7 mRNA and protein levels increased with aging only in the frontal lobe but not in the temporal lobe, occipital lobe, or the hippocampus of old female rats [18]. Therefore, SIRT7 expression shows variability not only among different tissues but also in diverse locations of a tissue.

Our results on decreased SIRT7 mRNA and protein expression in the liver of old rats are in accordance with similar findings in old rats [7] and mice [8]. Similar age-related changes in SIRT7 expression were reported upon cell senescence or aging in other models. Nucleolar SIRT7 protein level decreased in primary human fibroblasts undergoing replicative senescence [19], while the Sirt7 mRNA level decreased in HSCs of old as compared to young mice [7, 20].

Our finding of decreased Sirt7 gene expression in the cardiac muscle of old rats extends the previously published data obtained in aging mice [6]. The importance of SIRT7 for the heart muscle is related to its role in the regulation of mitochondrial function [8], control of p53-dependent apoptosis of cardiomyocytes and stress responses [6], including the response to acute cardiovascular injury in mice [21]. Thus, SIRT7 could prevent the aging-associated deterioration of cardiac muscle function [22–24].

For the first time, we demonstrated that aging decreases Sirt7 gene expression in rat skeletal muscles – both EDL, containing mainly fast-twitch glycolytic (white) fibers, and SOL, which is composed mainly of slow-twitch oxidative (red) fibers [25]. However, this change in mRNA was not accompanied by diminished SIRT7 protein level. Because SIRT7 was demonstrated to regulate the ubiquitin-proteasome system [26], responsible also for muscle protein degradation in old animals [27], we suggest that SIRT7 might function against sarcopenia, i.e. the decrease in skeletal muscle mass that occurs during physiological aging [22].

We also present novel data on SIRT7 expression upon SCR, which has not been previously studied. It was previously demonstrated that along with reduced body weight and WAT depot mass, SCR decreased blood serum triglycerides [15], whose level depends in part on hepatic lipogenic activity. Although SIRT7 is involved in the control of lipogenesis in the liver [9], we observed no effect of SCR on hepatic SIRT7 expression. Our finding contrasts with the results obtained in different models of nu-
tritional challenge. SIRT7 protein levels increased after 24-hour fasting in the liver of young mice [8], as well as upon nutrient deprivation in cultured HEK293T cells [10]. In contrast, the transient decrease in Sirt7 gene expression which we observed after 2-day-long refeeding in the liver of old rats can be explained by the inhibitory effect of feeding on SIRT7 expression [8]. Because SIRT7 deficiency was shown to result in liver steatosis in knock-out mice [8, 9], we speculate that the significant inhibition of SIRT7 in the old but not young animals’ liver tissue may contribute to greater fat accumulation upon over-feeding in the old than in the young liver.

We analyzed SIRT7 expression in WAT. Based on the phenotype of Sirt7 knockout mice, which had substantially less subcutaneous adipose tissue than their wild-type counterparts [6], and the SIRT7 control of lipogenesis in hepatocytes [8, 9], we hypothesized that SIRT7 may also be involved in lipid metabolism in adipose tissue. Therefore we analyzed SIRT7 expression upon SCR and refeeding, which were previously demonstrated to induce the activity of lipogenic enzymes in major WAT deposits [15]. Although we observed no significant alterations in SIRT7 expression in WAT after SCR, this result does not preclude SIRT7 involvement in the control of adipocyte metabolism. Importantly, an inhibitory effect of feeding on Sirt7 expression [8] was noted in the rWAT of old animals after 2-day-long refeeding. This observation underscores the heterogeneity of WAT depots, among which the retroperitoneal (also referred to as perirenal) adipose tissue is generally characterized by the highest metabolic activity and responsiveness to stimuli [15, 17].

Given the beneficial effects of long-term calorie restriction on the heart muscle diastolic function [28], and the suggested cardioprotective role of SIRT7 [24], we also analyzed SIRT7 expression in the heart of short-term calor-}

**References**


