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Growth attenuation is associated with histone deacetylase 10-induced autophagy in the liver

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Abstract

Our previous data suggested that the histone deacetylase (HDAC) SIRT1 is involved in mediating the effect of nutrition on growth. The aim of the present research was to study the mechanism by which additional HDACs may be involved in nutrition-induced linear growth. The *in vivo* studies were performed in young male Sprague–Dawley rats that were either fed *ad libitum* (AL) or subjected to 10 days of 40% food restriction (RES) and then refed (CU). For *in vitro* studies, Huh7 hepatoma cells were used. Food restriction led to significant reduction in liver weight, concomitant with increased autophagy (i.e., a decrease in the level of P62 and an increase in the expression level of Ambra1 and Atg16L2 genes in the RES group). At the same time, we found that the level of HDAC10 was significantly increased. Overexpression of HDAC10 in Huh7 hepatoma cells led to reduced cell viability and increased autophagy as shown by increased conversion of LC3-I to LC3-II. An increase in the level of HDAC10 was also obtained when mTOR was inhibited by Rapamycin. siRNA directed against HDAC10 abolished the effect of Rapamycin on cell viability and Ambra1 and Atg16L2 increased expression. These results suggest that increased levels of HDAC10 may mediate the effect of malnutrition on growth attenuation and autophagy. Deciphering the role of epigenetic regulation in the nutrition–growth connection may pave the way for the development of new forms of treatment for children with growth disorders.
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1. Introduction

Children's growth is regulated by both genetic and environmental factors. The most effective environmental factor known today is nutrition; however, the mechanisms connecting nutrition and longitudinal growth are still not fully understood. Several studies have shown a connection between linear growth and epigenetic mechanisms during chondrogenic differentiation [1–3]. Epigenetics is defined as changes in gene function caused by mechanisms other than changes in the genomic DNA sequence, such as methylation of the DNA as well as posttranslational modification of the histone proteins [4]. These may include phosphorylation, methylation, ubiquitinylation, sumoylation and acetylation [5,6]. The most extensively studied epigenetic modification of histones is acetylation. Histone acetylation by histone acetyltransferases (HATs) occurs at the ε-amino group of lysine residue within the peptide chain. The already bulky lysine side chain becomes even bulkier, the positive charge is neutralized and the histone–DNA interaction is weakened. Acetylation is cleared by histone deacetylases (HDACs). As a general rule, histone acetylation by HATs is usually associated with increased transcription activity because of the "loose" chromatin structure. Deacetylation by HDACs leads to condensation and suppression of transcription [7]. HATs and HDACs also use nonhistone protein substrates, including transcriptional regulators, chromatin components and signaling factors, adding another level of regulation [8–11].

There are 18 HDACs in the mammalian genome, divided into four groups according to their homology to yeast HDACs, their size, cellular localization, catalytic domain and mechanism of action. There are 11 zinc-dependent HDACs {class I (HDAC1, HDAC2, HDAC3 and HDAC8); class II, subdivided into class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10); and class IV (HDAC11) [12,13]} and 7 nicotinamide adenine dinucleotide (NAD+)-dependent HDACs, which comprise class III (sirtuin, SIRT1 to SIRT7). Class I HDACs are generally located in the nucleus and are relatively small in size; class II HDACs are present both in the nucleus and cytoplasm and are generally larger. Several HDACs were shown to be stimulated by food restriction; others were shown to be affected by a high-fat diet [14], indicating their sensitivity to the metabolic status and suggesting a possible role in the nutrition–growth connection. The most extensively studied HDACs in this context are the sirtuins, which deacetylate a number of histone and nonhistone substrates [15–17].

To study how malnutrition affects linear growth, we used a protocol of 40% food restriction for 10 days followed by refeeding with the same

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chow for 1 day, in young, fast-growing male Sprague–Dawley (SD) rats [18]. We previously found that SIRT1 of the HDAC III family is significantly increased in the epiphyseal growth plate (EGP) by food restriction, probably as a result of reduction in the level of miR-140 and miR-22, which target it [17]. Furthermore, we found that the effect of food restriction on the level of these miRNAs is mediated by leptin and the insulin-like growth factor IGF-1 as well as other as yet-unidentified systemic factors [19]. These findings prompted us to investigate whether there might be parallel effects in other tissues and indeed we found that the weight of the liver was rapidly affected by nutritional manipulation, similar to the EGP. This led to the present study to determine if HDACs could also be involved in food-restriction-induced growth attenuation in the liver.

In the present study, we investigated the role of the HDACs in the liver and its effect on growth and autophagy during food restriction. We found that food restriction significantly increased the protein levels of several HDACs in the liver. Our results suggest that HDAC10 responds to the nutritional status through mTOR and that it may be involved in autophagy induced by food restriction.

2. Materials and methods

2.1. Antibodies

Anti-HDAC1, HDAC6 and HDAC10 were obtained from Abcam (Cambridge, MA, USA); anti-HDAC10 was obtained also from Biovision (Milpitas, CA, USA); anti-HDAC4 and antimicrotubule-associated protein light chain LC3 were from Cell Signaling (Danvers, MA, USA); anti-HDAC5, HDAC7, HDAC8 and HDAC11 as well as anti-SQSTM1/P62 and anti-Hsp70/Hsc70 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-HDAC2 and anti-HDAC3 were from SAB Signalway Antibody (Baltimore, MD, USA); anti-HDAC9 was from Epitomics (Burlingame, CA, USA); anti-β-actin was from Millipore (Billerica, MA, USA); anti-FLAG were from Sigma-Aldrich (Rehovot, Israel); secondary fluorescent antibodies were obtained from LI-COR Biosciences (Lincoln, NE, USA).

2.2. Animals

Male SD rats, 24 days old, were purchased from Harlan (Jerusalem, Israel) and housed individually at the animal care facility of the Felsenstein Medical Research Center. The rats were divided into two groups: one was given an unlimited amount of food (complete diet for rats and mice, 3.4 kcal/g, provided by Teklad) (*ad libitum* group — AL, $n=6$), and the other one was given 60% of the same chow (food-restricted group — RES). All animals had unlimited access to water. The 40% restriction was calculated on the basis of a previous study wherein animals were housed individually and the amount of food consumed each day was measured, together with the animal's weight and weight gain [18]. The food restriction was maintained for 10 days. At that point, the RES group was further divided into two groups: one was kept restricted (RES, $n=6$), and the other one was given normal chow *ad libitum* (which induced catch-up growth; hence, this group was named CU group; $n=6$). After 1 day of refeeding, animals from all three groups were sacrificed by CO_2 inhalation; serum was collected and stored at −80°C until further use; and internal organs (kidney, lungs, liver and heart) were removed, weighed and immediately frozen in liquid nitrogen until analyzed. Part of each liver was stored in 4% formaldehyde and was later used for paraffin sections. Throughout the study, animals were observed daily, and all remained bright, alert and active, with no evidence of any disorder. The Tel Aviv University Animal Care Committee approved all procedures.

2.3. Huh7 cells

Huh7 cells were cultured in Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine and penicillin/streptomycin (all cell culture materials were from Biological Industries, Beit Haemek, Israel). Cells were grown in a humidified incubator at 37°C with 95% air/5% CO_2, and the medium was changed every other day.

2.4. Protein extraction and determination

Huh7 human hepatoma cells (washed with PBS) and rat livers were homogenized in a radioimmunoprecipitation assay buffer (20 Mm Tris–HCl, 150 mM NaCl, 1% NP-40 and 0.25% Na-deoxycholate) that was supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland) in a 1:12 ratio. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Illinois, USA) according to the manufacturer's recommendations, and 50 μg proteins was analyzed per each sample by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western immunoblotting. Proteins were transferred to nitrocellulose membranes (GE Healthcare, New Jersey, USA) by the wet blotting system (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes were then incubated in TBS-T (10 mM Tris–HCl, 150 mM NaCl and 0.1% Tween 20) with 5% skim milk solution for 1 h to block nonspecific binding and then incubated with the primary antibody overnight at 4°C. Membranes were washed with TBS-T, incubated with a secondary fluorescent antibody (LI-COR Biosciences, Lincoln, NE, USA) decorated with IRDye for 1 h and washed again. β-Actin served as the reference. Quantification was performed using the Odyssey (2.1.) application Software (LI-COR Biosciences, Lincoln, NE, USA).

2.5. RT^2 profiler rat autophagy PCR array

Total RNA was extracted from liver tissues of AL, RES and CU groups with miReasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The quantity and quality of the RNA were evaluated using a Nanodrop spectrophotometer (Nanodrop Ltd, USA) with values of $A_{260}/A_{280}>2.0$ and $A_{260}/A_{230}>1.7$. Equal amounts of RNA from all groups were used for analysis by the Rat Autophagy RT^2 Profiler PCR Array (SABiosciences, USA), which profiles the expression of 84 genes related to autophagy. cDNA was synthesized with RT^2 First Strand Kit (SABiosciences, USA), using 600 ng of total RNA, preceded by DNase I treatment. Using real-time PCR, the expression of a focused panel of genes was measured according to the manufacturer's recommendations, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Results were confirmed by real-time PCR reactions performed with specific RT^2 quantitative PCR primer assay/SYBR Green Rat for AMBRA1 and ATG16L2 genes (SABiosciences, USA); RPLP1 (ribosomal protein large, P1) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) served as the internal controls. The following thermal cycling conditions were used: one cycle at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method [20]. Each sample was examined in duplicates, and three biological samples were tested per each point.

2.6. Transient transfection

FLAG-HDAC10 plasmid was kindly provided by Dr. Hung-Ying Kao from the Case Western Reserve University School of Medicine, Cleveland, OH, USA [21]. As a negative control and for transfection efficiency, we used the pEGFP-C1 vector expressing a green fluorescent protein (GFP) [17]. Successful transfection was validated by following GFP expression under a fluorescent microscope. Cells were transfected using the TransIT-LT1 (Mirus, Madison, WI, USA) according to the manufacturer's recommendations.

For the knockout experiment, we used a set of four siRNAs specifically designed to silence HDAC10: siRNA #1 (ID 2150743), #2 (ID 2150744), #4 (ID 2150745) and #5 (ID 2150746) (all designed and provided by Qiagen, Valencia, CA, USA). AllStars Neg. siRNA AF 488 (Qiagen) was used as a negative control (NC). This time cells were transfected using the HiPerFect (Qiagen) as the transfection reagent. All transfection protocols were performed on Huh7 cells grown in 24- and 96-well plate format according to the manufacturer's instructions.

2.7. Assessment of Huh7 cell viability

Huh7 cells were plated in 96-well plates (cell culture clusters, polystyrene; Corning, Tewksbury, MA, USA); cell viability was determined by adding 10% Alamar Blue, a metabolic indicator dye (Alamar Blue assay; AbD Serotec, Raleigh, NC, USA), to each well together with the culture medium and incubating the plates at 37°C for 4 h. Medium absorbance was measured with a fluorimetric reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), with excitation at 544 nm and emission at 590 nm. The fluorescence in the study groups is expressed as a percentage of the fluorescence in the control group.

2.8. PAS (periodic acid Schiff) staining protocol for glycogen detection

The PAS staining kit was obtained from Merck (Whitehouse Station, NJ, USA) and was used on paraffin sections of rat liver from all experimental groups ($n=3$ per each group) according to the manufacturer's protocol.

2.9. Folch method for lipid content determination

Chloroform/methanol in a relative volume of 2:1 was added to homogenized 100 mg of liver tissue. After dispersion, the whole mixture was agitated during 15–20 min on an orbital shaker at room temperature. The homogenate tissue was centrifuged to recover the liquid phase, and then the solvent was transferred to a clean tube, washed with 200 μl water and vortexed for a few seconds to obtain two separate phases. The lower phase was transferred to a new tube and air dried. The amount of lipid extract that was dried in the tube was weighed and calculated per 100 mg of tissue [22].

2.10. Immunoprecipitation

Total cell lysates were incubated overnight with anti-Hsp70/Hsc70 antibody followed by 30 min incubation with protein A/G microbeads. The immunocomplexes were washed and eluted using MultiMACSTM Protein A/G Kit according to the manufacturer's protocol (MACS Miltenyi Biotec, BG, Germany).

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