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Lifelong calorie restriction affects indicators of colonic health in aging C57Bl/6J mice☆

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Abstract

Diminished colonic health is associated with various age-related pathologies. Calorie restriction (CR) is an effective strategy to increase healthy lifespan, although underlying mechanisms are not fully elucidated. Here, we report the effects of lifelong CR on indicators of colonic health in aging C57Bl/6J mice. Compared to an *ad libitum* control and moderate-fat diet, 30% energy reduction was associated with attenuated immune- and inflammation-related gene expression in the colon. Furthermore, expression of genes involved in lipid metabolism was higher upon CR, which may point towards efficient regulation of energy metabolism. The relative abundance of bacteria considered beneficial to colonic health, such as *Bifidobacterium* and *Lactobacillus*, increased in the mice exposed to CR for 28 months as compared to the other diet groups. We found lower plasma levels of interleukin-6 and lower levels of various metabolites, among which are bile acids, in the colonic luminal content of CR-exposed mice as compared to the other diet groups. Switching from CR to an *ad libitum* moderate-fat diet at old age (24 months) revealed remarkable phenotypic plasticity in terms of gene expression, microbiota composition and metabolite levels, although expression of a subset of genes remained CR-associated. This study demonstrated in a comprehensive way that CR affects indicators of colonic health in aging mice. Our findings provide unique leads for further studies that need to address optimal and feasible strategies for prolonged energy deprivation, which may contribute to healthy aging.

Keywords: Aging; Calorie restriction; Colonic health; Gene expression; Gut microbiota; Metabolites

1. Introduction

Aging is characterized by a time-dependent accumulation of cellular and molecular damage, which in turn results in various age-related morbidities [1–3]. To date, the most effective strategy to improve both health and lifespan in various model organisms is calorie restriction (CR), which refers to a reduced energy intake (usually 30%–40% restriction) without malnutrition [4,5]. Also for humans, emerging evidence suggests that CR is associated with physiological, metabolic and molecular adaptations that may, at least partly, contribute to improved metabolic and molecular health [6].

The mechanisms underlying the effects of CR are, despite extensive research, only partially understood. A complex interplay between metabolic adaptations, immune responses and other molecular processes is thought to be responsible for the health-promoting and life-extending effects of CR [7,8]. Attenuation of chronic low-grade and age-related inflammation, also called inflammaging [9], is considered a plausible mechanism via which CR prevents or delays common pathologies such as cancer, cardiometabolic diseases and cognitive decline [10–13].

Emerging evidence shows that a disturbed balance between beneficial and harmful bacteria is one of the driving forces behind inflammaging [14,15]. From this perspective, the colon plays a pivotal

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role in maintenance of health during aging as the intestinal epithelial barrier function and permeability determine interaction with the intestinal microbiota and the external environment [16,17]. We have previously shown that aging is accompanied by changes in expression of genes related to inflammation and enhanced immune responses in the colon of aging mice [18]. Also, age-related changes in microbiota composition and bacterial metabolites, including short chain fatty acids, have been described in aging populations of rodents and humans [15,19–21].

Interestingly, it has been shown that CR is able to alter the intestinal microbiota composition in aging mice [22]. The potential of CR to suppress detrimental processes in the colon, such as carcinogenesis [23,24], further emphasizes that CR is a plausible and powerful candidate that may support maintenance of colonic health during aging. However, detailed insight into persistence of CR-induced effects upon challenging conditions is scarce [25]. It has been demonstrated that favorable metabolic effects are persistent after discontinuation of prolonged CR [26,27]. To what extent this "metabolic memory" also applies to the colon and whether CR-induced effects on markers of colonic health are persistent is not known.

In this study, we provided a comprehensive overview of the molecular, microbial and metabolic effects of lifelong CR, referring to 30% energy reduction without malnutrition, in the colon. The effects of CR were contrasted against a control diet (C) and a Western-style moderate-fat (MF) diet. Microbiota composition, whole-genome gene expression profiles, levels of metabolites and circulating cytokines were determined in male C57BL/6J mice at young (6 months) and old age (28 months). At 24 months of age, a subgroup of mice was transferred from CR to an *ad libitum* MF diet (CR>MF). This diet switch provided the unique opportunity to study phenotypic plasticity in CR-exposed mice.

2. Materials and methods

2.1. Animals and diets

The design of the study and experimental procedures have been described in detail previously [28,29]. Briefly, male C57BL/6J mice were individually housed and randomized to different dietary regimens at the age of 9 weeks. For the current analyses, we considered mice that were lifelong fed a semisynthetic control diet consisting of 10E% of fat (C), a CR diet without malnutrition (30% energy reduction compared to the C diet) or an MF diet consisting of 25E% of fat (Supplementary Table A1). All diets, except the CR diet, were fed *ad libitum*. Mice sacrificed at 6 or 28 months of age were considered in order to differentiate between effects at young and old age. To study the molecular flexibility and dynamics after receiving a strict CR diet for prolonged time, a diet switch was included in this study. A subgroup of mice on CR (n=16) was switched to the MF diet at the age of 24 months until sacrifice at 28 months.

Upon sacrifice at 6 or 28 months of age, colon and plasma samples were collected for 11–18 mice per intervention group. The mice were fasted for 4 h prior to sacrifice after which they received an intragastric gavage of either solvent (mock: 0.5% carboxymethyl cellulose) or Wy-14643 dispersed in solvent (160 mg Wy-14643/kg body weight) and were then fasted again for another 6 h. The purpose of the treatment with Wy-14643, which is a PPARα agonist, was to examine PPARα adaptive capacity in the liver, which has been covered in a separate publication [30]. Since Wy-14643 can cause fast and pronounced effects on gene expression in various organs [31,32], only mock-treated mice were considered for the gene expression analyses. For the analyses of the microbiota composition and plasma interleukin-6 (Il6) levels, Wy-treated mice were additionally included to increase the statistical power. Results on food intake, body weight development, survival characteristics and liver transcriptome data of the mice have been published elsewhere [33].

At sacrifice, blood samples were collected by cardiac puncture. The colon was opened longitudinally, and colonic luminal contents were obtained by flushing fecal pellets with phosphate-buffered saline (PBS) and immediately stored at −80°C. The colonic luminal content samples were used for metabolite and microbiota profiling. Scrapings of the mucosa and submucosa from the distal and proximal segments of the colon were collected, snap frozen in liquid nitrogen and stored at −80°C for whole-genome gene expression profiling. At the age of 28 months, urinary samples were collected on consecutive days until a total of 1 ml was obtained. Individual mice were held above a clean and empty Petri dish, and the urine was transferred to an Eppendorf tube which was stored at −80°C. The design of the study and the experiments described were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University (drs-2010151b).

2.2. Circulating levels of cytokines

Plasma levels of Il6 were determined at 6 and 28 months of age (n=8–17 mice per diet group) using the customized Mouse Adipokine Magnetic Bead Panel MILLIPLEX MAP assay (kit MADKMAG-71K, Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Data were log (base2) transformed for visualization and statistical analyses.

2.3. RNA isolation and gene expression analyses

To determine the transcriptional effects of the different dietary regimens, whole-genome gene expression profiles were studied in the colonic scrapings of mice at 6 and 28 months of age. Colonic samples from 44 mice, ranging from 5 to 7 mice per group, were analyzed. Total RNA was isolated from the scrapings using TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Breda, the Netherlands). Isolated RNA was purified using RNeasy Micro columns (Qiagen, Venlo, the Netherlands), and total RNA yield (Nanodrop ND-1000, Nanodrop Products, Maarssen, the Netherlands) and RNA integrity (Agilent 2100 Bioanalyzer, Agilent Technologies, Amsterdam, the Netherlands) were assessed for all samples.

Purified RNA (100 ng per sample) was converted to cDNA and labeled using an Ambion WT expression kit (Life Technologies, Bleiswijk, the Netherlands). Samples were hybridized to the Affymetrix GeneChip Mouse Gene 1.1 ST array according to the standard Affymetrix protocols (Affymetrix, Santa Clara, CA, USA). Two samples did not pass the quality control and were excluded, resulting in a total of 42 samples with microarray data available. The arrays were normalized using the Robust Multichip Average (RMA) approach [34]. Probe sets were defined according to Dai et al. [35] using the chip description file version 19.0.0 based on the Entrez Gene database. Genes that had an RMA expression value >20 in at least 5 arrays were considered expressed. Only those genes with an interquartile range >0.1 for all samples together were included in the analyses, resulting in a total of 16,071 genes. Differences in gene expression between the diet groups were analyzed using the Intensity Based Moderated T statistics (IBMT) [36] with P values <.01 as threshold. Microarray data are available from the Gene Expression Omnibus repository with number GSE100701.

2.4. Determination of the microbiota composition based on 16S rRNA gene profiling

Microbiota composition was determined in the colonic luminal content of 8–10 mice per diet group at the age of 6 and 28 months, resulting in a total of 68 samples. Genomic DNA was extracted from the colonic luminal content using the ZR Fecal DNA MicroPrep kit (ZYMO Research, Irvine, CA, USA) according to the instructions of the manufacturer. Subsequently, the V3-V4 region of the 16S ribosomal RNA (rRNA) gene was amplified using a 2-step polymerase chain reaction (PCR). First, 10–25 ng of DNA was amplified using the 341F (5′-CCTACGGGNGGCWGCAG-3′) and 785R (5′-GACTACHVGGGTATCTAATCC-3′) primers appended with Illumina adaptor sequences. Amplicons were purified using the ZR-96 DNA Clean and Concentrator-5 Kit (ZYMO Research, Irvine, CA, USA). To quantify DNA content of the resulting amplicon, the Qubit Fluorometric Quantitation device (ThermoFisher Scientific, Waltham, MA, USA) was used. Besides, the amplicons were run on a 2% agarose gel to confirm amplicon size and amount. Purified PCR products were used for the second PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina, San Diego, CA, USA). Purified PCR products with an amplicon length of 550 base pairs (bp) (including adaptors) were submitted for sequencing in the paired-end (2×) modus (300 bp) on the MiSeq platform (Illumina, San Diego, CA, USA) at the BaseClear service laboratory (BaseClear BV, Leiden, the Netherlands). FASTQ files were generated and demultiplexed based on sample-specific barcodes using the Casava pipeline (version 1.8.3, Illumina, San Diego, CA, USA). Sequence reads of low quality (only "passing filter" reads were selected) or containing the adapters or PhiX control signals were removed, and the FASTQC pipeline (version 0.10.0, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was applied for the initial quality control. One sample was excluded (old mouse on MF diet) because the coverage was below the threshold (50% of the median number of reads).

Further preprocessing of the sequencing data, including merging of the paired reads and fixed length trimming of the reads (cutoff is mean length of the merged reads minus 1 standard deviation), was performed using the CLC Microbial Genomics Module (version 1.2.1, CLC Bio, Qiagen, Aarhus, Denmark) in the CLC Genomics Workbench (version 8.5.1, CLC Bio, Qiagen, Aarhus, Denmark). Based on a total of 763,543 reads, 1017 operational taxonomic unit (OTU) groups were identified after alignment against the Silva database [37] at 97% similarity. For 31 of these OTUs (225 reads in total), taxonomic annotation could not be retrieved (N/A). Differences in relative abundances for the diet or age groups were determined using a Kruskal–Wallis test with Bonferroni correction for multiple comparisons (adjusted P<.05). Dunn's *post hoc* comparisons (adjusted P value <.05) were performed to further explore the contrasts and identify genera that differed between the diet groups at young or old age.

The CLC Microbial Genomics Module was also used to determine the alpha and beta diversities. For these analyses, all OTUs with a combined number of ≥10 reads were considered. The Shannon entropy was used as a measure for alpha diversity (richness and evenness). The beta diversity, showing differentiation of the taxa among the different samples, was determined using Bray–Curtis distances. Individual samples were visualized in corresponding principal coordinate analyses (PCoAs) plots for the first two principal