Influences of aging and caloric restriction on the transcriptional profile of skeletal muscle from rhesus monkeys

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In laboratory rodents, caloric restriction (CR) retards several age-dependent physiological and biochemical changes in skeletal muscle, including increased steady-state levels of oxidative damage to lipids, DNA, and proteins. We have previously used high-density oligonucleotide arrays to show that CR can prevent or delay most of the major age-related transcriptional alterations in the gastrocnemius muscle of C57BL/6 mice. Here we report the effects of aging and adult-onset CR on the gene expression profile of 7,070 genes in the vastus lateralis muscle from rhesus monkeys. Gene expression analysis of aged rhesus monkeys (mean age of 26 years) was compared with that of young animals (mean age of 8 years). Aging resulted in a selective up-regulation of transcripts involved in inflammation and oxidative stress, and a down-regulation of genes involved in mitochondrial electron transport and oxidative phosphorylation. Middle-aged monkeys (mean age of 20 years) subjected to CR since early adulthood (mean age of 11 years) were studied to determine the gene expression profile induced by CR. CR resulted in an up-regulation of cytoskeletal protein-encoding genes, and also a decrease in the expression of genes involved in mitochondrial bioenergetics. Surprisingly, we did not observe any evidence for an inhibitory effect of adult-onset CR on age-related changes in gene expression. These results indicate that the induction of an oxidative stress-induced transcriptional response may be a common feature of aging in skeletal muscle of rodents and primates, but the extent to which CR modifies these responses may be species-specific.

Caloric restriction (CR) extends maximum lifespan and retards the development of a broad spectrum of pathophysiological changes in laboratory rodents (1). We have previously used high-density oligonucleotide arrays to demonstrate that aging is associated with specific transcriptional alterations in the gastrocnemius muscle, cerebral cortex, and cerebellum of C57BL/6 mice and that CR can prevent or delay most of the largest age-related transcriptional alterations (2, 3). These studies suggest that monitoring the effects of CR on multiple tissues is feasible through the use of DNA microarrays. Regarding human health, investigation of the ability of CR to retard aging and diseases in a long-lived nonhuman primate species may be more germane than rodent studies. A long-term CR study in adult (8- to 14-year-old) male rhesus monkeys, the maximum lifespan of which is ≈40 years, was initiated in 1989 at the Wisconsin Regional Primate Research Center (4). This ongoing study, along with a similar study at the National Institute on Aging (5), provides opportunities to investigate the ability of adult-onset CR to influence longevity, disease patterns, and other consequences of biological aging in primates.

To examine the molecular events associated with aging in this nonhuman primate species and the influence of CR on these events, we used oligonucleotide-based arrays to define the transcriptional response to the aging process in vastus lateralis muscle. Our choice of tissue was guided by skeletal muscle being primarily composed of long-lived, high oxygen-consuming postmitotic cells, a feature shared with other critical aging targets such as heart and brain. Also, we have previously used oligonucleotide microarrays to investigate the influences of aging and CR in mouse skeletal muscle (gastrocnemius) (2). The loss of skeletal muscle mass during aging, often referred to as sarcopenia, leads to frailty and is of great public health significance but of unknown etiology (6).

Methods

Animals and Experimental Design. This study involved 12 male rhesus monkeys (*Macaca mulatta*), born and housed indoors with complete clinical and experimental histories available. The influence of aging was studied in two groups ($n = 3$) of conventionally maintained monkeys given free access to Purina (St. Louis, MO) Monkey Chow (no. 5038). These monkeys were either young (7–11 years old) or old (25–27 years old). For the CR study, normally fed ($n = 3$) and calorie-restricted ($n = 3$) middle-aged (19–21 years old) monkeys from our long-term CR study were examined. These animals had been subjected to CR for 9 years when biopsies of vastus lateralis were obtained for this study. No animal had any clinical or experimental history expected to differentially affect skeletal muscle. This protocol was carried out with the approval of the Institutional Animal Care and Use Committee of the University of Wisconsin.

The methods used to house and feed the animals are described in detail elsewhere (4, 7). To summarize briefly for the long-term CR study, both control and calorie-restricted animals were fed a purified diet (no. 85387, Teklad, Madison, WI) and housed individually for accurate measurement of daily food intake, but they had extensive auditory and visual contact with other monkeys housed in the same room. For all monkeys, temperature was maintained at ≈21°C with average relative humidity of 50–65%. Room lighting was automatically controlled to provide alternating 12-hr periods of light and darkness. The CR animals had ≈30% lower food intakes than the controls. This restriction was achieved by randomly assigning animals to a treatment group after a 3- to 5-month period of baseline assessment, during which food intake of the experimental diet was determined for individual animals. Food intake of the animals assigned to CR was reduced from their baseline period averages by 10% per month for 3 months and then maintained at this 30% restriction level. The controls continued to have free access to food. In 1994, the CR monkeys were switched to the caloric equivalent of a modified semipurified diet (Teklad no. 93131), which is enriched by 30% in vitamins and minerals.

Tissue Preparation for DNA Microarray Analysis. Details for tissue preparation and procedures for the use of Affymetrix microar-

GENETICS

Abbreviations: OXPHOS, oxidative phosphorylation; CR, caloric restriction.

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Table 1. Genes up-regulated with aging in vastus lateralis muscle

ORF	Fold (Old/Young)	SE	Gene	Function
U29615	7.5	2.1	Chitotriosidase	Macrophage factor
L17328	5.5	2.6	Pre-T/NK cell associated protein	T/NK cell development
M30894	4.7	1.1	T cell receptor gamma locus	T-cell antigen receptor
D31797	4.6	1.8	CD40 ligand	B-cell proliferation
X62320	3.9	0.7	Granulin	Inflammation, wound repair
X16663	3.9	1.0	Hematopoietic cell-specific Lyn substrate 1	Antigen receptor signaling
X58529	3.8	0.5	Immunoglobulin mu	B-cell development
M18737	3.7	0.5	Granzyme A	Target cells lysis
U11877	3.7	0.7	IL-8 receptor type B	IL8 receptor
U35005	3.3	0.7	MAPK 8	Inflammatory signal transduction
Z11697	3.2	0.7	CD83	Lymphocyte activation
M64174	3.0	0.3	JAK1	Interleukin signal transduction
X85786	2.9	0.5	Regulatory factor X, 5	Regulation of MHC class II gene
M36634	2.9	0.8	Vasoactive Intestinal Peptide	Cytokine modulation
U50136	2.9	1.0	Leukotriene C4 synthase	Allergic inflammation
M26683	2.8	0.5	Monocyte chemotactic protein	Monocyte recruitment
M37435	2.7	0.7	CSF-1	Cytokine activation in hematopoiesis
U19713	2.6	0.5	Allograft-inflammatory factor-1	Macrophage activation
M62486	2.6	0.7	Complement C4b-binding protein	Regulation of complement system
D90276	2.5	0.5	CGM7	Immunoglobulin
X03934	2.4	0.4	CD3, delta	T cell receptor signal transduction
D49950	2.4	0.5	IL-18	Cytokine
X68733	2.3	0.6	Antichymotrypsin,alpha1	Immune response modifier
L37036	2.1	0.2	neutrophil-activating peptide 78	Inflammatory chemokine
Z29572	2.1	0.2	B-cell maturation factor	B-cell maturation
U26710	2.1	0.5	Cas-Br-M	Lymphocyte maturation
U09579	4.1	0.6	P21	DNA damage response
U65785	4.1	0.9	Oxygen-regulated protein 150	Chaperone
M20867	3.6	0.7	Glutamate dehydrogenase	Ammonia detoxification
M11717	3.4	0.6	Heat shock protein 70	Chaperone
J00123	3.3	1.5	Proenkephalin	Pain perception
Y00498	3.1	0.7	Cytochrome P450 IIC8	Detoxification
M13485	3.0	0.5	Metallothionein I B	Protection against oxidative stress
U50648	3.0	0.7	Interferon-inducible RNA-dependent protein	Interferon-inducible protein
X76538	2.9	0.4	Mpv17	Reactive oxygen metabolism
U77845	2.5	0.7	TRIP	NF-kappa-B modulation
M87499	2.4	0.2	Uracil-DNA glycosylase	DNA repair
M29874	2.3	0.4	Cytochrome P450 IIB	Detoxification
M10943	2.2	0.3	Metallothionein I F	Protection against oxidative stress
U38964	2.2	0.3	PMSR2	DNA repair
M61855	2.2	0.5	Cytochrome P450 IIC9	Detoxification
L11005	2.1	0.3	Aldehyde oxidase	Detoxification
U33838	2.1	0.4	NF-kappa-B p65	Oxidative stress response
X13930	2.0	0.3	Cytochrome P450 IIA6	Detoxification
M62302	5.5	1.4	Growth/differentiation factor 1	Neuronal development
L12260	3.9	0.9	Neuregulin 1	Myelin repair
D83699	2.9	0.4	Neuronal death protein	Apoptosis activation
M17409	2.8	0.6	NCAM1	Outgrowth of neurites
U79716	2.8	0.8	Reelin	Neuronal migration
X99664	2.7	0.7	SH3GL3	Neuronal cell death
U13706	2.3	0.4	Hel-N2	Neuron-specific RNA-binding protein
U66702	2.0	0.2	Phogrin	Neuronal development
X77343	2.0	0.4	Transcription factor AP-2 alpha	Neurogenesis

Inflammatory Response Stress Response Neuronal Factors

rays have been described (2). Total RNA was isolated from each biopsy and individual samples were used for gene expression profiles. Target RNA was prepared by converting 1 μg of mRNA into double-stranded cDNA (Superscript Choice System, GIBCO/BRL) with a T7-$(dT)_{24}$ primer incorporating a T7 RNA polymerase promoter. Biotin-labeled cRNA was synthesized from cDNA by using an RNA transcript labeling kit (Enzo Biochem). After complementary RNA had been fragmented to sizes ranging from 35 to 200 bases by heating (35 min at 95°C), 10 μg of RNA fragments were hybridized (16 h at 45°C) to a HuGeneFL Array (Affymetrix, Santa Clara, CA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics system. The chips were scanned with a Hewlett Packard GeneArray Scanner.

Data Analysis. The Affymetrix HuGeneFL Array contained about 7,070 human genes and expressed sequence tags (ESTs) from UniGene (Build 18), GenBank, and The Institute for Genomic Research (TIGR) databases. Each gene was represented in the array by 20 perfectly matched (PM) oligonucleotides and 20 mismatched (MM) control probes that contain a single central-base mismatch. Fluorescence intensity was read for each oligonucleotide to calculate the average signal intensity (SI) for each gene by subtracting the intensities of ≈20 PM oligonucleotides from the intensity of the MM control probes, after discarding the maximum, the minimum, and any outliers beyond three standard deviations. All calculations were performed by an Affymetrix algorithm. To determine the effects of aging, nine pairwise comparisons between young (n = 3) and old (n = 3) individuals were performed. Likewise, the effects of CR were determined by comparing each calorie-restricted (n = 3) to each control (n = 3) monkey, generating nine pairwise comparisons. The data reported in Tables 1–5 and in Tables 6–9, which are published as supplemental data on the PNAS web site, www.pnas.org, represent the average fold changes obtained through the nine pairwise combinations. To be acceptable for reporting, average fold changes had to exceed 1 SE from a 1.3-fold change.

Statistical Analysis of the Effect of CR on Changes in Gene Expression. A statistical analysis was conducted for a subset of 34 genes that were either up-regulated or down-regulated in both aged and middle-aged monkeys. The analysis asked whether there was evidence of any effect of CR on expression level by regressing expression level on dummy codes for both CR (0 = no CR; 1 = CR)

Table 2. Genes down-regulated with aging in vastus lateralis muscle

ORF	Fold (Old/Young)	SE	Gene	Function
Y10812	-8.3	6.1	Fructose 1,6-bisphosphatase 2	Gluconeogenesis
U90915	-7.9	6.7	Cytochrome c oxidase subunit IV	Mitochondrial OXPHOS
D26308	-7.8	3.8	Flavin reductase	Unknown
X76057	-6.4	1.4	Phosphomannose isomerase	Glycosylation
X79537	-6.2	4.2	Glycogenin	Glycogen synthesis
M22538	-4.1	2.3	NADH-ubiquinone reductase	Mitochondrial OXPHOS
X15341	-3.9	1.2	Cytochrome c oxidase subunit VIa	Mitochondrial OXPHOS
J05401	-3.7	1.0	Sarcomeric mitochondrial creatine kinase	Energy transduction
L08666	-3.7	1.6	VDAC 2	Voltage-dependent anion channel
M20681	-3.5	1.5	GLUT3	Glucose transporter
X96752	-3.4	0.8	L-3-hydroxyacyl-CoA dehydrogenase	Mitochondrial beta-oxidation
U17886	-3.4	1.5	Succinate dehydrogenase iron-protein subunit	Mitochondrial OXPHOS
D16562	-3.2	1.2	ATP synthase gamma-subunit (L-type)	Mitochondrial OXPHOS
X84195	-2.9	0.9	Acylphosphatase	Hydrolysis of acylphosphates
U09813	-2.6	0.8	Mitochondrial ATP synthase subunit 9	Mitochondrial OXPHOS
D55654	-2.5	1.1	Malate dehydrogenase 1	Citric acid cycle
U59309	-2.3	0.4	Fumarase precursor	Citric acid cycle
U27460	-2.2	0.4	Uridine diphosphoglucose pyrophosphorylase	Glucosyl donor
J04823	-2.2	0.4	Cytochrome c oxidase subunit VIII	Mitochondrial OXPHOS
Z35093	-2.1	0.6	Surfeit 1	Mitochondrial OXPHOS
D14710	-2.0	0.4	ATP synthase alpha	Mitochondrial OXPHOS
M37104	-2.0	0.4	Mitochondrial ATPase coupling factor 6	Mitochondrial OXPHOS
Y00764	-2.0	0.5	Mitochondrial hinge protein ubiquinol-cytochrome c	Mitochondrial OXPHOS
Z19585	-13.2	5.3	Thrombospondin-4	Nervous system development
M60278	-8.5	3.9	Diphtheria toxin receptor	Cellular proliferation
X87843	-8.3	2.8	Cyclin H assembly factor	Cell cycle control
J05272	-5.7	4.0	IMP dehydrogenase type 1	Cell growth regulation
M57710	-5.2	1.5	Galectin 3	Cell growth
J03040	-4.9	3.0	Osteonectin	Cell proliferation, repair
D83783	-4.8	1.3	Thyroid hormone receptor-associated protein, 230-Kd	Early fetal development
M80359	-4.4	2.7	MAP/ microtubule affinity regulating kinase 3	Cell cycle regulation
U15655	-4.3	0.8	Ets2 repressor factor	Transcriptional factor
L19182	-4.0	1.4	Insulin-like growth factor binding protein 7 (Mac25)	Growth-suppressing factor
S54005	-4.0	1.7	Thymosin beta-10	Unknown
X61755	-3.9	2.0	Homeoprotein HOX3D	Development
U18291	-3.5	0.9	Cell division cycle 16, homolog	Cell mitosis control
L27560	-2.7	0.6	Insulin-like growth factor binding protein 5	Control of IGF action
X00588	-2.3	0.4	EGF receptor	Cell proliferation
Z69030	-2.1	0.5	PP2A gamma 1 isoform	Cell proliferation
D87461	-4.9	1.7	BCL2-like 2	Prevention of apoptosis
X16832	-4.1	1.0	Cathepsin H	Protein degradation
X65965	-3.8	2.0	Manganese superoxide dismutase	Mitochondrial antioxidant
U39817	-3.6	0.6	Bloom syndrome protein	DNA ligase activity, DNA repair
AF005043	-2.8	0.8	Poly(ADP-ribose) glycohydrolase	DNA repair
L48513	-2.5	0.4	Paraoxonase 2	Unknown
J04810	-2.5	0.5	MSH3	DNA mismatch repair
U10117	-2.4	0.4	Endothelial-monocyte activating polypeptide II	Cellular activation

Energy Metabolism Growth Regulatory Protein Stress Response

and Age (0 = young; 1 = middle-aged) by ordinary least-squares linear regression.

Results

Human High-Density Oligonucleotide Arrays Can Be Used to Monitor Gene Expression Patterns in Rhesus Monkeys. To determine whether Affymetrix HuGeneFL arrays could be used to detect transcriptional changes in rhesus monkeys, we compared hybridization patterns of human and monkey vastus lateralis samples. From a total of 7,070 represented cDNAs, the number of transcripts resulting in positive signal intensities was 4,895 for humans ($n = 5$) and 4,677 for rhesus monkeys ($n = 5$). Average intragroup correlation coefficients were lower in the rhesus monkeys (0.83, $n = 3$) as compared with humans (0.92, $n = 5$). These observations indicate that the degree of sequence identity between humans and rhesus monkeys allows for oligonucleotide-based microarray analysis of rhesus monkeys by using human arrays, a finding that should be applicable to other nonhuman primate species.

Age-Related Changes in Gene Expression. Comparison of hybridization patterns from the vastus lateralis of young adult (7–11 years old) and old (25–27 years old) monkeys revealed that aging is associated with a 2-fold or higher increase in expression of 300 genes, 4.2% of the total number of genes represented in the oligonucleotide array (for a full list of the 300 genes, see Table 6, which is published as supplemental data on the PNAS web site, www.pnas.org). Of these genes, 24 (7.9%) encode proteins that could be linked to an inflammatory/immune function as determined by database searches, representing the largest class of transcripts that display a large (2-fold or more) change in expression pattern with aging (Table 1). The transcript displaying the largest change in expression (7.5-fold) was chitotriosidase (Table 1). Chitotriosidase is a member of the chitinase family of proteins which are secreted by activated human macrophages (8) and is markedly elevated in plasma of Gaucher disease, a disorder characterized by the presence of large amounts of activated, lipid-laden macrophages in spleen, liver, and other tissues (9). Genes involved in B cell function included CD40 ligand, immunoglobulin μ chain, and B cell maturation factor. Genes involved in inflammation include granulin, allograft-inflammatory factor-1, neutrophil-activating peptide 78, and leukotriene C4 synthase. We also observed the concerted induction of genes involved in an oxidative stress response, including HSP-70, the 150-kDa oxygen-regulated protein ORP150, and the p65 subunit of NF-κB. Additionally, genes involved in neuronal death, remodeling, and repair were consistently activated, including reelin, glial growth factor-2 (neuregulin 1), and phogrin. This latter observation is consistent with the loss of motor neuron units and subsequent re-innervation of muscle fibers during aging in mammals (10, 11).

One hundred and forty-nine (2.1%) genes were down-regulated

Table 3. Genes up-regulated by CR in vastus lateraris muscle

ORF	Fold	SE	Gene	Function
Z74616	8.2	2.8	Collagen I, alpha 2	Extracellular matrix
S81737	7.0	2.0	Syntrophin, alpha 1	Binds to nNOS in muscle
M60832	5.8	0.9	Collagen VIII, alpha 2	Extracellular matrix
X06700	5.1	1.7	Collagen III, alpha 1	Extracellular matrix
M63391	5.1	1.8	Desmin	Intermediate filament
M55998	4.3	1.2	Collagen I, alpha 1	Extracellular matrix
M13299	3.9	0.8	Blue cone photoreceptor pigment	Visual pigments
X01703	3.8	0.7	Tubulin, alpha, brain-specific	Microtubule component
X53416	3.8	2.2	Filamin A, alpha	Actin filament crosslink
X62515	3.7	1.1	Heparan sulfate proteoglycan 2	Basement membrane protein
X02761	3.7	1.1	Fibronectin 1	Cell adhesion
X82207	3.5	1.5	Centractin, beta	Microtubule component
X52022	3.1	0.8	Collagen VI, alpha 3	Extracellular matrix
X05855	3.1	1.2	Histone H3.3	Nucleosome structure
X72012	3.0	0.7	Endoglin	Endothelial cell glycoprotein
D38583	2.8	0.4	Calgizzarin	Cytoskeletal assembly
Z26653	2.8	0.6	Laminin, alpha 2	Basement membranes
U21128	2.8	0.6	Lumican	Interactions with collagen
X14474	2.7	0.8	Microtubule-associated tau protein	Microtubule component
S80562	2.6	0.5	Calponin 3, acidic	Smooth muscle contraction
D14446	2.6	0.6	Fibrinogen-like 1	Fibrinogen-related gene
M69181	2.5	0.6	Myosin, heavy polypeptide 10	Cytokinesis
M31013	2.5	1.2	Myosin, heavy polypeptide 9	Cytokinesis
D78367	2.4	0.6	K12 keratin	Epithelium maintenance
M10277	2.1	0.2	Actin, beta	Cell motility
J05243	2.1	0.4	Spectrin, alpha, non-erythrocytic 1	Actin crosslink
X73039	8.5	3.9	Sox12	Unknown
U52101	4.5	1.1	Epithelial membrane protein 3	Cell-cell interactions
D63391	4.0	1.3	Platelet activating factor acetylhydrolase IB γ subunit	Neuronal development
D38305	3.2	1.4	Tob	Cell growth suppression
M80563	2.8	0.5	CAPL	Calcium binding
L13698	2.8	0.7	Growth arrest-specific 1	Cell growth suppression
M19720	2.7	0.6	L-myc	Cellular proliferation
M62403	2.7	1.4	Insulin-like growth factor binding protein 4	Inhibits IGF action
S59184	2.2	0.4	RYK receptor-like tyrosine kinase	Growth factor receptor
U09278	2.2	0.4	Fibroblast activation protein	Fibroblast growth
J02763	2.1	0.3	Calcyclin	Calcium binding protein
D50310	2.1	0.4	Cyclin I	Unknown
S53175	9.0	1.2	NADH-ubiquinone oxidoreductase, 51 Kda Subunit	Mitochondrial OXPHOS
M19878	5.4	1.8	Calbindin 1	Ca(2+)-ATPase stimulation
U54617	5.3	3.0	Pyruvate dehydrogenase kinase isoform 4	Glucose metabolism
X13916	4.0	0.9	LDL receptor related protein 1	Lipoprotein metabolism
M28713	2.0	0.4	NADH-cytochrome B5 reductase	Glycolysis

Structural Protein Growth Regulatory Protein Energy Metabolism

with aging in the vastus lateralis muscle of rhesus monkeys (see Table 7, which is published as supplemental data on the PNAS web site, www.pnas.org). These included genes involved in energy metabolism, cell growth, structural components, and stress response (Table 2). Striking among these was the abundance of genes related to energy metabolism, accounting for 15.8% of all down-regulated genes. These included several nuclear encoded proteins that function in mitochondrial bioenergetics, such as the sarcomeric mitochondrial creatine kinase, NADH:ubiquinone reductase, ATP synthase α and γ subunits, and cytochrome *c* oxidase subunit VIa. Recent studies strongly suggest that cytochrome *c* oxidase controls mitochondrial energy metabolism (12).

Effect of Adult-Onset CR on Gene Expression Profiles. To determine the effect of CR on gene expression profile, we compared the transcriptional patterns of middle-aged monkeys (mean age of 20 years) receiving the control diet to aged-matched middle-aged monkeys that been subjected to CR since early adulthood. This comparison allows for the identification of transcriptional shifts that are diet-related, as opposed to age-related, and therefore represent a transcriptional reprogramming induced by CR. The expression of 107 genes (1.5%) was up-regulated by CR by 2-fold or more (for a full listing of these genes, see Table 8, which is published as supplemental data on the PNAS web site, www.pnas.org). The major transcriptional class induced by CR was composed by genes encoding structural proteins (Table 3). These included several collagens (collagen I α1 and α2 subunits, collagen VII α2 subunit, and collagen III α1 subunit). The extracellular matrix of muscle is composed mostly of collagens, and collagen synthesis in skeletal muscle is up-regulated by growth factors such as growth hormone (13, 14). In contrast, collagen genes are down-regulated in streptozotocin-induced diabetes in rats (15). Other cytoskeletal or extracellular matrix-encoding genes induced by CR include desmin, laminin, β-actin, and myosin heavy chain. CR also induced the expression of several genes that appear to have roles in cellular growth, such as L-myc, insulin-like growth factor binding protein 4, cyclin 1, and calcyclin (Table 3).

CR down-regulated 93 genes (1.3%) by 2-fold or more (see Table 9, which is published as supplemental data on the PNAS web site, www.pnas.org). The major transcriptional class in this group of genes encodes proteins involved in energy metabolism (Table 4). The largest decrease in expression (23-fold) was observed for cytochrome c_1, a component of the electron-transport chain complex III. Interestingly, cytochrome c_1 expression is extremely sensitive to thyroid status at both mRNA (16) and protein levels (17). Other alterations in gene expression linked to oxidative phosphorylation (OXPHOS) include the genes that encode the mitochondrially located cytochrome *c* oxidase subunit VII, cytochrome *c* subunit IV, sarcomeric mitochondrial creatine kinase, ATP synthase α and β subunits, and ubiquinol:cytochrome *c* reductase core protein II. Other energy-related transcripts reduced in expression by CR include fructose-1,6-bisphosphatase, malate dehydrogenase, NADH:ubiquinone oxidoreductase, and NADH reductase. These transcriptional alterations provide support for the concept that CR monkeys may be in a hypometabolic state associated with reduced activity of the mitochondrial electron transport system.

Effect of Adult-Onset CR on Age-Associated Alterations. To investigate the effect of adult-onset CR on age-associated alterations

Table 4. Genes down-regulated by CR in vastus lateraris muscle

ORF	Fold	SE	Gene	Function
J04444	-22.8	12.7	Cytochrome c-1	Mitochondrial OXPHOS
U16660	-8.1	2.9	Peroxisomal enoyl-CoA hydratase-like protein	Peroxisomal beta-oxidation
Y10812	-5.6	2.3	Fructose-1,6-bisphosphatase 2	Gluconeogenesis
X76057	-3.9	2.0	Phosphomannose isomerase	Glycosylation reactions
U90915	-3.6	0.5	Cytochrome c oxidase subunit IV	Mitochondrial OXPHOS
U40490	-2.8	0.8	Nicotinamide nucleotide transhydrogenase	Mitochondrial proton pump
J04823	-2.4	0.7	Cytochrome c oxidase subunit VIII	Mitochondrial OXPHOS
D16294	-2.3	0.6	Mitochondrial 3-oxoacyl-CoA thiolase	Mitochondrial beta-oxidation
J05401	-2.2	0.3	Sarcomeric mitochondrial creatine kinase	Energy transduction
J04973	-2.2	0.4	Ubiquinol-cytochrome c reductase core protein II	Mitochondrial OXPHOS
D55654	-2.1	0.3	Malate dehydrogenase 1	Citric acid cycle
X96752	-2.1	0.3	L-3-hydroxyacyl-CoA dehydrogenase	Mitochondrial beta-oxidation
M22538	-2.1	0.4	NADH ubiquinone reductase	Mitochondrial OXPHOS
M22632	-2.0	0.2	Mitochondrial aspartate aminotransferase	Amino acid metabolism
M19483	-2.0	0.2	ATP synthase beta subunit	Mitochondrial OXPHOS
D16481	-2.0	0.3	Mitochondrial 3-ketoacyl-CoA thiolase B-subunit	Mitochondrial beta-oxidation
L08666	-2.0	0.3	Voltage-dependent anion channel 2	Mitochondrial anion channel
M93284	-2.0	0.4	Pancreatic lipase related protein 2	Lipid metabolism
M26730	-2.0	0.6	Ubiquinol-cytochrome c reductase binding protein	Mitochondrial OXPHOS
X62744	-4.1	2.2	MHC, class II, DM alpha	Class II antigen presentation
X13810	-3.1	1.2	Octamer transcription factor 2	Ig gene activation
D29675	-4.2	0.7	Inducible nitric oxide synthase	Nitric oxide synthesis
X07619	-3.2	1.8	Cytochrome P450 IID7A	Detoxification
D87075	-2.9	0.8	L-ascorbic acid transporter 2	ROS metabolism
D89858	-2.0	0.4	D-aspartate oxidase	Oxidative deamination

Energy Metabolism Inflammatory Response Stress Response

in gene expression, we obtained muscle biopsies from rhesus monkeys that had been subjected to CR since early adulthood. The control group consisted of animals that had the same mean age and consumed a similar, but higher-calorie, semipurified diet since early adulthood. We first determined whether changes in gene expression observed in older monkeys (Tables 1 and 2) were either completely or partially established in middle-aged monkeys. We identified 34 transcripts that were elevated or decreased in expression with aging, and also by middle age (Table 5). The vast majority (32/34) displayed an intermediate level of expression between young and old animals.

Next, we determined how many of such transcripts were altered in expression as a result of caloric restriction. A statistical analysis was conducted for this subset of genes. The analysis asked whether there was any evidence of any effect of CR on expression level by regressing expression level on dummy codes for both CR (0 = no CR; 1 = CR) and Age (0 = young; 1 = middle aged) by ordinary least-squares linear regression. Surprisingly, only three genes ap-

Table 5. Effects of CR on age-associated changes in gene expression in middle-aged rhesus monkeys

ORF	OC/YC	SE	MC/YC	SE	M CR/YC	SE	CR Effect	Gene	Function
L17328	5.5	2.6	4.0	2.8	6.5	3.5	NS	Pre-T/NK cell associated protein	T/NK cell development
M30894	4.7	1.1	2.5	0.5	4.1	1.3	NS	T-cell receptor gamma locus	T-cell antigen receptor
X62320	3.9	0.7	3.4	1.3	5.0	0.4	NS	Granulin	Inflammation, wound repair
U11872	3.7	0.7	4.8	2.3	6.8	1.6	NS	IL-8 receptor type B	IL8 receptor
M64174	3.0	0.3	2.7	0.7	3.1	0.4	NS	JAK1	Interleukin signal transduction
D49950	2.4	0.5	2.0	0.5	2.6	0.7	NS	IL-18	Immune responses
U65785	4.1	0.9	3.0	0.6	3.7	0.4	NS	Oxygen-regulated protein 150	Chaperone
Y00498	3.1	0.7	2.6	0.5	2.6	0.6	NS	Cytochrome P450 IIC8	Detoxification
X76538	2.9	0.4	2.5	0.4	2.7	0.4	NS	Mpv17	Reactive oxygen metabolism
M87499	2.4	0.2	1.9	0.3	2.1	0.1	NS	Uracil-DNA glycosylase	Uracil removal from DNA
M13485	3.0	0.5	2.1	0.3	2.1	0.3	NS	Metallothionein IB	Protection against oxidative stress
M29874	2.3	0.4	2.4	0.5	2.7	0.3	NS	Cytochrome P450 IIB	Detoxification
M10943	2.2	0.3	1.6	0.2	1.9	0.1	NS	Metallothionein IF	Protection against oxidative stress
M62302	5.5	1.4	3.3	1.1	6.1	1.5	NS	Growth/differentiation factor 1	Neuronal development
L12260	3.9	0.9	1.8	0.9	2.6	0.8	NS	Neureglin 1	Myelin repair
X14675	5.3	1.1	2.2	0.9	2.9	1.0	NS	BCR-ABL	Myeloid leukemia induction
X15341	-3.9	1.2	-1.7	0.3	-1.7	0.3	NS	Cytochrome c oxidase subunit VIa	Mitochondrial OXPHOS
U17886	**-3.4**	**1.5**	**-1.4**	**0.1**	**-2.3**	**0.3**	**0.03**	**Succinate dehydrogenase iron-protein subunit**	**Mitochondrial OXPHOS**
X96752	-3.4	0.8	-1.7	0.3	-3.3	0.5	NS	L-3-hydroxyacyl-CoA dehydrogenase	Mitochondrial beta-oxidation
D49387	**-3.1**	**1.6**	**-1.8**	**0.2**	**-4.9**	**2.2**	**0.01**	**Leukotriene B4 12-hydroxydehydrogenase**	**Inactivation of leukotriene B(4)**
X84195	-2.9	0.9	-1.6	0.2	-2.0	0.2	NS	Acylphosphatase	Hydrolysis of acylphosphates
U27460	-2.2	0.4	-2.0	0.9	-2.3	0.6	NS	Uridine diphosphoglucose pyrophosphorylase	Glucosyl donor
Y00764	-2.0	0.5	-1.4	0.6	-2.1	0.3	NS	Mitochondrial hinge protein ubiquinol-cyt. c reductase	Mitochondrial OXPHOS
Z19585	-13.2	5.3	-6.1	3.9	-2.2	1.4	NS	Thrombospondin-4	Nervous system development
M57710	-5.2	1.5	-3.9	1.4	-4.4	1.8	NS	Galectin 3	Cell growth
U15655	-4.3	0.8	-1.9	0.8	-3.8	1.4	NS	Ets repressor factor	Transcriptional factor
S54005	-4.0	1.7	-3.9	2.5	-0.9	1.4	NS	Thymosin beta-10	Unknown
L19182	-4.0	1.4	-2.1	0.4	-2.1	0.5	NS	Insulin-like growth factor binding protein 7	Growth-suppressing factor
U18291	-3.5	0.9	-2.1	0.6	-2.7	0.6	NS	Cell division cycle 16, homolog	Cell mitosis control
M95787	-7.7	2.8	-7.3	4.7	-5.1	5.0	NS	Transgelin	Smooth muscle contraction
D38037	-5.1	1.0	-4.1	0.6	-8.1	2.9	NS	FK506-binding protein 1B	Excitation-contraction
M55683	**-4.1**	**1.1**	**-2.6**	**0.8**	**-5.0**	**1.0**	**0.01**	**Cartilage matrix protein**	**Collagen binding**
M19267	-2.1	0.4	-2.1	0.5	-2.1	0.8	NS	Tropomyosin	Muscle contraction
D87461	-4.9	1.7	-3.9	1.9	-3.9	1.9	NS	BCL2-like 2	Cell survival, anti-apoptosis

OC, old control; YC, young control; MC, middle-aged control; M CR, middle-aged CR; NS, not significant. Significant CR effects are are printed in red.

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peared to show an effect, and it represented aggravation of down-regulations observed with aging (i.e., CR further reduced the level of expression). The two-tailed P values were U17886, $P = 0.029$; D49387, $P = 0.027$; and M55683, $P = 0.009$. These genes encoded, respectively, succinate dehydrogenase iron-protein subunit, a component of the complex II of the mitochondrial electron transport chain (18), and leukotriene B_4 12-hydroxydehydrogenase, which converts leukotriene B_4, a potent chemotactic and proinflammatory factor, into its biologically less active metabolite, 12-oxoleukotriene B_4 (19), and a cartilage matrix protein (matrilin-1) (20).

Discussion

This large-scale gene expression analysis in a nonhuman primate underscores the potential of human high-density oligonucleotide arrays to monitor gene expression profiles of nonhuman primates. Because the DNA sequence homology between humans and rhesus monkeys is likely to be in the range of 95–98%, these results are not unexpected. In fact, a previous study used human high-density oligonucleotide array-based analysis to determine the distant history of single-nucleotide polymorphisms in chimpanzee, pygmy chimpanzee, and gorilla genomic DNA samples (21).

The analysis of age-associated alterations in gene expression in the vastus lateralis muscle of rhesus monkeys is consistent with our previous study in the gastrocnemius muscle of mice that demonstrated an age-associated induction of genes involved in stress responses and lowered expression of metabolic and biosynthetic genes (2). In particular, we have determined that transcripts induced by reactive oxygen species, such as oxygen-regulated protein 150, HSP70, metallothioneins IB and IIF, and NF-κB are up-regulated in aging skeletal muscle of rhesus monkeys (Table 1). This finding agrees with a recent study from our group that used electron microscopic (EM) techniques with antibodies raised against 4-hydroxy-2-nonenal (HNE)-modified proteins, dinitrophenol, and nitrotyrosine to quantify and localize the age-dependent accrual of oxidative damage in rhesus monkey vastus lateralis skeletal muscle. Using ImmunoGold EM analysis of muscle from rhesus monkeys ranging in age from 2 to 34 years old, we observed a 4-fold maximal increase in levels of HNE-modified proteins. Likewise, carbonyl levels increased approximately 2-fold with aging (22). The decrease in expression of transcripts involved in OXPHOS in aged rhesus monkeys is also in agreement with our previous observations in mice. Possibly, the reduction in gene expression of metabolic genes is due to an accrual of oxidative damage over the lifespan, leading to reduced mitochondrial function or biogenesis.

The effects of CR on gene expression profiles are striking, with a strong increase in the expression of structural and cytoskeletal genes and a reduction in the expression of mitochondria-related genes. Previous studies have determined that the expression of extracellular matrix genes in muscle is strongly modulated by growth factors such as growth hormone (13, 14). Therefore, it is plausible that alterations in hormonal levels may underlie the observed changes in gene expression. A recent study that has used high-density oligonucleotide arrays to investigate the gene expression profile of diabetes in mice has revealed that high glucose levels and poor insulin sensitivity result in lowered expression of cytoskeletal genes in adipocytes (Alan D. Attie, personal communication). In contrast, animals in our study display lower serum glucose levels and increased insulin sensitivity (23). An interesting observation of our study is the decrease in expression of genes involved in OXPHOS function in both aged animals receiving the control diets and middle-aged CR animals. We postulate that such alterations have a distinct molecular basis and are induced by mitochondrial dysfunction in aged animals as opposed to lower metabolic rate in CR animals. In particular, the 23-fold lower expression of cytochrome c_1, which is particularly sensitive to thyroid status (16, 17), was observed in CR monkeys (Table 4) but not in aged animals (Table 2). Consistent with this hypothesis, long-term (2-year) CR leads to reduced energy expenditure and sleeping metabolic rate in humans (24). The concept that reduced OXPHOS gene expression activity with aging is caused by mitochondrial and metabolic dysfunction is supported by the induction of reactive oxygen species and inflammatory related transcripts in aged skeletal muscle of rhesus monkeys (Table 1), and by the accrual of oxidative damage (22) and high levels of mitochondrial DNA deletions in skeletal muscle of these animals (25).

Surprisingly, we did not observe beneficial effects of adult-onset CR on the progression of age-related transcriptional markers (Table 5). This finding is particularly striking, because this animal cohort is clearly showing the physiological beneficial effects of CR, such as increased insulin sensitivity, reduced blood glucose (23), and reduced oxidative stress-induced cytokine expression by peripheral blood mononuclear cells (26). This finding stands in contrast to our previous report that more than 80% of age-related changes in gene expression found in skeletal muscle are either partially or completely suppressed by early-onset CR in mice (2). Possibly, aging retardation at the transcriptional level can be observed only if CR is initiated early in life, or if tissues are profiled late in life. Alternatively, the ability of CR to suppress changes in gene expression associated with aging may be species-specific. The latter hypothesis suggests that despite beneficial effects on hormonal parameters and possible increases in survival rates, aging rates are not retarded at the molecular level by CR in primates. Testing these possibilities will require studies employing early-onset CR in primates and the examination of multiple tissues for age-related changes in gene expression in CR and control animals.

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