

Symposium: Calorie Restriction: Effects on Body Composition, Insulin Signaling and Aging

Microarray Profiling of Gene Expression in Aging and Its Alteration by Caloric Restriction in Mice^{1,2}

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ABSTRACT An active research area in biological gerontology concerns the mechanisms by which caloric restriction (CR) retards the aging process in laboratory rodents. We used high density oligonucleotide arrays representing 6347 genes to determine the gene expression profile of the aging process in gastrocnemius muscle of male C57BL/6 mice. Aging resulted in a differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes. Most alterations were completely or partially prevented by CR. Transcriptional patterns of muscle from calorie-restricted animals suggest that CR retards the aging process by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage. The use of high density oligonucleotide microarrays provides a new tool to measure biological age on a tissue-specific basis and to evaluate at the molecular level the efficacy of nutritional interventions designed to retard the aging process. *J. Nutr.* 131: 918S–923S, 2001.

KEY WORDS: • caloric restriction • aging • gene expression • oligonucleotide microarrays • muscle

Caloric restriction (CR)⁴ retards the aging process in laboratory mice and rats (Weindruch and Walford 1988, Fishbein 1991, Yu 1994). This retardation of aging by CR is manifested by a delayed occurrence or complete prevention of a broad spectrum of age-associated pathophysiological changes and a 30–50% increase in maximum life span. CR also lengthens the maximum life span of fish, rotifers, spiders and other nonmammals (Weindruch and Walford 1988).

Because no other intervention has been shown to retard the aging process in mammals, an active research area in biological gerontology concerns the mechanisms by which CR retards aging in laboratory rodents. These are challenging studies because CR induces hundreds (if not hundreds of thousands)

of biological changes, making it difficult to identify those that are causal. Five classes of interrelated and nonexclusive explanations for the mechanism of CR are: 1) decreases in oxidative stress (Sohal and Weindruch 1996); 2) decreases in glycation or glycoxidation (Kristal and Yu 1992); 3) decreases in body temperature and circulating thyroid hormone levels associated with a hypometabolic state (Walford and Spindler 1997); 4) alterations in gene expression and protein degradation (Van Remmen et al. 1995); and 5) neuroendocrine changes (Nelson et al. 1995).

This brief article discusses changes in gene expression induced by CR in laboratory mice. We first discuss earlier work using Northern hybridization and then summarize our recent study (Lee et al. 1999) of mouse gastrocnemius muscle using oligonucleotide microarrays. The latter approach provides a powerful tool to evaluate nutritional interventions at the transcriptional level because it produces a broad assessment of gene expression patterns.

Gene expression changes induced by CR as measured by Northern hybridization

A summary of CR-induced changes in mRNA levels in mice as measured by Northern hybridization is shown in **Table 1**. Most information is available for liver. Studies from Stephen Spindler's laboratory suggest that CR may lower enzyme capacity for glycolysis and increase enzyme capacity for gluconeogenesis and disposal of by-products of muscle protein

¹ Presented at the symposium "Calorie Restriction: Effects on Body Composition, Insulin Signaling and Aging" as part of the Experimental Biology 2000 meeting held April 15–18, 2000 in San Diego, California. This symposium was sponsored by the American Society for Nutritional Sciences and the American Society for Clinical. The proceedings of this symposium are published as a supplement to *The Journal of Nutrition*. Guest Editor for this supplement publication was Barbara Hansen, Obesity and Diabetes Research Center, School of Medicine, University of Maryland, Baltimore, Maryland.

² Supported by National Institutes of Health Grants P01 AG11915 (to R.W.), R01 AG18922 (to R.W.) and R01 CA78723 (to T.A.P.). T.A.P. is a recipient of the Shaw Scientist (Milwaukee Foundation), Burroughs Wellcome Young Investigator and Basil O'Connor (March of Dimes) awards.

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⁴ Abbreviations used: CR, caloric restriction; PM, perfect matches; MM, mismatch; ROS, reactive oxygen species.

TABLE 1

Gene expression changes induced by caloric restriction in mice as measured by Northern hybridization

Strain	Ages-Sex	Tissue	Gene and CR effect	Reference
C3B10RF ₁	7, 28-F	Liver	↑ 1.7–2.3 G6Pase; ↑ 2.5 PEPCK: (28 mo); ↑ 2.4 glutaminase; ↑ 1.4–1.8 carbamyl phosphate synthase I, TAT; pyruvate kinase, glutamine synthetase	Dhahbi et al. 1999
C3B10RF ₁	7, 28-F	Muscle Liver	↑ 1.3–2.1 glutamine synthetase ↓ 1.3–1.5; ERp57, ERp72, GRP170, GRP78, GRP 94, calreticulin (28 mo)	Dhahbi et al. 1997
C3B10RF ₁	21-F	Liver	NSD: GRP75, HSC70, PDI, C/EBP alpha	Dhahbi et al. 1998
Emory	5, 22-F, M	Liver	NSD catalase 4.5 mo: ↓ 1.4–1.5 ubiquitin, catalase, Gpx; NSD: Cu/Zn, Mn-SOD 22 mo: ↑ 1.7 ubiquitin	Mura et al. 1996
C3B10RF ₁	21-F	Liver	NSD: Cu/Zn, Mn-SOD, catalase, Gpx	Tillman et al. 1996a
C3B10RF ₁	33-F	Liver	↑ 3.0 carbamyl phosphate synthetase I: ↓ 1.7 GRP78	Tillman et al. 1996b
C3B10RF ₁	5, 17, 30-F	Liver	↓ 1.4–1.5 GRP 78 and GRP94	Spindler et al. 1990
R3/Sa	2–9-F	Breast Pituitary	↓ 4.0–15.0 MMTV NSD prolactin	Li et al 1994
C3H/Ou	7–9-F	Breast	↓ 4.0–5.0 MMTV	Engelman et al. 1991
AKR	1.5-F	Thymus	↓ 1.7 MCF MuLV	Shields et al. 1991
C3B10RF ₁	5, 17, 30-F	Liver	↑ 1.2 insulin receptor: NSD: glucocorticoid receptor, IGF-1, RNA, c-jun, Sp1, polymerase 2 EF, C/EBP:	Spindler et al. 1991
C3B10RF ₁	5, 17, 30-F	Liver	↑ 1.2–2.0: Cyt. P1- and P3-450, MnSOD; (30 mo) NSD: catalase, CuZn-SOD, GPx, EH	Mote et al. 1991

¹ Values are fold changes comparing calorie-restricted mice to controls. Ages are in months. NSD, no significant difference; F, female; M, male; PEPCK, phosphoenol pyruvate carboxykinase

catabolism. Also, the mRNA levels of hepatic endoplasmic reticulum chaperones were reduced by CR. Other studies show decreased expression of viral genes in calorie-restricted mice. The mRNA levels for several antioxidant enzymes, in most cases, were not influenced by CR. A striking feature of this dataset is the scarcity of information in brain, heart and other organs.

Oligonucleotide microarrays as a tool to evaluate nutritional interventions

DNA microarrays are likely to revolutionize biomedical research through the simultaneous analysis of gene expression patterns of whole genomes (Lander 1999). Large-scale analysis of transcriptional responses has already proven to be useful in the analysis of complex biological phenomena such as metabolism in *Saccharomyces cerevisiae* (DeRisi et al. 1997) and tumorigenesis in humans (Perou et al. 1999). Because such arrays are synthesized based on sequence information, they provide a direct link between differential gene expression patterns and information readily available in public genomic databases.

DNA microarrays can be broadly divided into two classes, cDNA-based and oligonucleotide-based arrays. cDNA-based arrays are simpler to construct, and several devices are commercially available that spot cDNAs onto glass slides (Brown and Botstein 1999). These slides can be used to quantify mRNA abundance after hybridization with tissue-specific cDNA pools obtained through reverse transcription of polyA mRNA. A disadvantage of this approach is that a large fraction of mammalian genes are members of gene families, and family members can easily cross-hybridize, causing spurious results. In contrast, oligonucleotide arrays contain tens of thousands of gene-specific oligonucleotides synthesized in situ using light-directed, solid-phase combinatorial chemistry (Lipshutz et al. 1999). This technology was introduced by Af-

fymetrix (Santa Clara, CA) whose arrays contain 40 specific oligonucleotide probes, which are 25 base pairs long, for each gene represented in the array. Twenty of these oligonucleotides represent perfect matches (PM probes) to the gene of interest along its length. Another 20 oligonucleotides contain a single mismatch (MM probes) compared with the PM probes. To calculate an average signal intensity, hybridization intensity from each MM probe is subtracted from that of its PM counterpart and the values are averaged for the 20 pairs.

The gene expression profile of aging and CR in mouse gastrocnemius muscle

To examine the molecular events associated with aging in mammals, we used oligonucleotide-based arrays to elucidate the transcriptional response to the aging process in mouse gastrocnemius muscle (Lee et al. 1999). Skeletal muscle was studied because it is primarily composed of long-lived, high oxygen-consuming postmitotic cells, a feature shared with other critical aging targets such as heart and brain. Loss of muscle mass (sarcopenia) and associated motor dysfunction is a leading cause of frailty and disability in older persons (Dutta and Hadley 1995).

The gene expression profile of aging. A comparison of gastrocnemius muscle from 5- and 30-mo-old male C57BL/6 mice revealed that aging is associated with alterations at the mRNA level, which may reflect changes in gene expression, mRNA stability or both. Of the 6347 genes surveyed in the oligonucleotide microarray, only 58 (0.9%) displayed a > two-fold increase in gene expression levels as a function of aging, whereas 55 (0.9%) displayed a > twofold decrease in expression. Therefore, gene expression patterns seem to be remarkably stable during the adult mammalian life span, a finding that contrasts with the hypothesis that aging is due to large and widespread alterations in gene expression.

Based on a literature search, functional classes were as-

signed and regulatory mechanisms inferred for specific sets of alterations. Of the 58 genes that increased > twofold in expression with age, 16% (9/58) could be assigned to stress responses. Genes in this category included the heat shock factors Hsp71 and Hsp27, protease Do and the DNA damage-inducible gene GADD45. Interestingly, the largest differential expression between young and aged animals (3.8-fold) was the mitochondrial sarcomeric creatine kinase, a critical target for peroxynitrite-induced inactivation (Stachowiak et al. 1998). Peroxynitrite is a strong oxidant generated in mitochondria through the reaction of superoxide with nitric oxide. Possibly, the induction of mitochondrial sarcomeric creatine kinase is a compensatory response to the increased production of reactive oxygen species (ROS) in mitochondria of aged animals (Sohal and Weindruch 1996). Taken as a whole, these observations provide support at the transcriptional level for a state of increased ROS production with aging, resulting in macromolecular damage.

A well-known consequence of both aging and muscle injury is reinnervation of motor neuron units (Larsson 1995). Genes involved in neuronal growth accounted for 9% (5/58) of genes highly induced in 30-mo-old animals, including neurotrophin-3 (Copray and Brouwer 1997), a growth factor induced during reinnervation, and synaptic vesicle protein-2, implicated in neurite extension (Marazzi and Buckley 1993). An age-associated disturbance in calcium homeostasis, which may play a major role in aging of the central nervous system, has been linked to increased activity of voltage-activated calcium channels (Disterhoft et al. 1994). Interestingly, we observed an increase in the expression of the dihydropyridine-sensitive L-type calcium channel in muscle from 30-mo-old animals, a finding that suggests altered calcium homeostasis in aged skeletal muscle. An increased catabolic state in aged muscle is suggested by the induction of the beta lysosomal protein-encoding gene, PEA3, a transcriptional factor induced in the response to muscle injury and previously shown to be highly expressed in muscle from old rats (Peterson and Houle 1997) was also induced in aged muscle. We also observed interesting parallels between our results and data obtained in fibroblasts undergoing *in vitro* replicative senescence. For example, HIC-5, an oxidative damage-induced transcriptional factor (Fujita et al. 1998) and insulin-like growth factor binding protein, both associated with *in vitro* senescence (Wang et al. 1996), are induced in aged skeletal muscle.

Taken as a whole, these results provide evidence that the aging process is characterized by the activation of an adaptive stress response consistent with increased production of ROS in aging muscle. Additionally, gene expression patterns suggest that secondary responses involve the activation of neuronal and myogenic responses to injury.

Transcriptional evidence for an age-related decline in metabolic capacity, biosynthesis and protein metabolism. Fifty-five of the 6347 genes assayed (0.9%) displayed a > twofold age-related decrease in expression. Genes involved in energy metabolism accounted for 13% (7/55) of these alterations. Several transcriptional alterations suggest a decrease in mitochondrial function or turnover, including reduced expression of the ATP synthase A chain and NADP transhydrogenase genes (both involved in mitochondrial bioenergetics), the LON protease implicated in mitochondrial biogenesis and the ERV1 gene involved in mtDNA maintenance. Presumably, this transcriptional pattern reflects either decreased mitochondrial biogenesis or turnover, secondary to cumulative ROS-inflicted mitochondrial damage (Sohal and Sohal 1991, Beckman and Ames 1998). These observations lend support to the concept that mitochondrial dysfunction plays a central

role in aging of postmitotic tissues. Additionally, a metabolic deficit is suggested by a decline in the expression of genes involved in glycolysis, glycogen metabolism and the glycerophosphate shunt.

Aging was also characterized by large reductions (twofold or more) in the expression of biosynthetic enzymes such as cytochrome P450 IIC12 (steroid biosynthesis), squalene synthase (fatty acid and cholesterol synthesis), stearoyl-CoA desaturase (polyunsaturated fatty acid synthesis) and EF-1-gamma (protein synthesis). This suppression in biosynthetic genes was accompanied by a concerted decrease in the expression of genes involved in protein turnover, such as the 20S proteasome subunit, the 26S proteasome component TBP1, ubiquitin-thiolesterase and the Unp ubiquitin-specific protease, all of which are involved in the ubiquitin-proteasome pathway of protein turnover (Schwartz and Ciechanover 1999). When taken as a whole, these observations suggest that as the induction of a stress response associated with damaged proteins and other macromolecules ensues, the very systems required for the turnover of such molecules are declining with age, perhaps due to an energetic deficit in the cell.

We also observed an intriguing and strong age-related downregulation (3.7-fold decrease) of a gene thought to be involved in gene silencing, MeCP2 [an abundant CpG binding protein (Nan et al. 1998)]. Although we have no clear understanding of the significance of this change, it is noteworthy due to the postulated role of DNA methylation alterations in aging (Cooney 1993) and cancer (Lengauer et al. 1997). In contrast to the consistent gene expression patterns observed for stress response genes (increased with age) and energy metabolism (decreased with age), the directions of changes in other categories, such as signal transduction, transcriptional and growth factors did not present a consistent age-related trend.

Age-related changes in gene expression are reversed by CR. If differential gene expression patterns in tissues of aged animals represent biological (as opposed to chronological) markers of organismal senescence, a significant fraction of these markers should be affected by CR, the only experimental intervention shown to extend the maximum life span of mammals (Weindruch and Walford 1988). Further, CR-mediated attenuation of specific age-related changes in gene expression would confirm the significance of the data obtained using high density oligonucleotide arrays and would validate their utility as markers of biological age.

To study the effects of CR on the gene expression profile of aging, caloric intake of C57BL/6 mice was reduced to 76% of control animals in early adulthood (2 mo of age), and this dietary regimen was maintained until the age of death at 30 mo. Age-related changes in gene expression profiles were remarkably attenuated by CR. Of the largest age-associated alterations (2.0-fold or higher), 29% (33/113) were completely prevented by CR and 34% (38/113) were partially suppressed. Of the four major gene classes that displayed consistent age-associated alterations (i.e., stress response, biosynthesis, protein metabolism and energy metabolism), 84% (26/31) were either completely or partially suppressed by CR. The striking effect of CR on age-related changes in gene expression patterns validates the conclusion that most of these reflect biological age and can serve as biomarkers of aging. Also, these observations imply that some molecular aspects of the aging process are markedly suppressed by CR, suggesting that elucidation of molecular mechanisms will provide opportunities for intervention.

Aging retardation by CR is associated with a metabolic shift. A global view of alterations in gene expression in CR

mice suggests a metabolic reprogramming characterized by a transcriptional shift toward energy metabolism, increased biosynthesis and protein turnover. CR resulted in the induction of 51 genes (1.8-fold or higher) compared with age-matched controls. Nineteen percent of genes (10/51) in this class are related to energy metabolism. Modulation of carbohydrate metabolism was evident through the induction of glucose-6-phosphate isomerase (glycolysis), fructose 1,6-bisphosphatase (gluconeogenesis), IPP-2 (an inhibitor of glycogen synthesis) and transketolase. Fructose 1,6-bisphosphatase switches the direction of a key regulatory step in glycolysis, reversing glycolysis toward a biosynthetic precursor, glucose-6-phosphate. Remarkably, this same adaptation has been observed as part of the transcriptional reprogramming of *Saccharomyces cerevisiae* accompanying the diauxic switch from anaerobic growth to aerobic respiration upon depletion of glucose (DeRisi et al. 1997). Transketolase, which controls the nonoxidative branch of the pentose phosphate pathway, provides NADPH for biosynthesis and reducing power for several antioxidant systems. CR also induced transcripts associated with fatty acid metabolism, such as fatty acid synthase and PPAR- δ , a mediator of peroxisome proliferation. Interestingly, CR may act to increase insulin sensitivity through the induction of glucose-dependent insulinotropic peptide and PPAR- γ , a potent insulin sensitizer (Zierath et al. 1998, Wu et al. 1999). Possibly, alterations in insulin signaling by CR in mice and through life-extending alleles of *Caenorhabditis elegans daf-2* (which encodes an insulin receptor) are mediated through a similar pathway that senses the energetic state of the cell (Ogg et al. 1997, Tissenbaum and Ruvkun 1998).

Perhaps due to the reduced availability of dietary sources, biosynthetic ability also seems to be induced in CR mice based on higher expression of glutamine synthase, purine nucleoside phosphorylase (purine turnover) and thymidylate kinase. Remarkably, 16% of transcripts (8/51) highly induced by CR encode proteins involved in protein synthesis and turnover, including elongation factor 1- γ , proteasome activator PA28, translocon-associated protein delta, 60S ribosomal protein L23 and the 26S proteasome subunit TBP-1. The overt shift in gene expression profile toward protein turnover and biosynthesis in CR mice contrasts with the evidence for reduced protein turnover and the accumulation of altered proteins in age-matched animals receiving the control diet. These findings agree with previous observations of a reduced level of oxidatively modified proteins in CR animals (Lass et al. 1998, Zainal et al., unpublished results) and provide transcriptional evidence for a central mechanism of action of CR in aging retardation of postmitotic tissues involving shifts in energy metabolism and increases in protein turnover.

Gene expression profile in calorie-restricted mice suggests decreased macromolecular damage. CR was associated with a 1.6-fold or greater reduction in expression of 57 genes. Twelve percent of these (7/57) were clearly associated with stress responses and/or DNA repair. The most substantial suppression of gene expression by CR among the 6347 genes examined was for a murine DnaJ homolog (3.4-fold), a pivotal and inducible heat shock factor that senses and transduces the presence of misfolded proteins in bacteria (Tomoyasu et al. 1998). Presumably, reduced generation of toxic by-products of metabolism in CR mice results in a lower steady-state level of modified proteins, triggering a down-regulation of this gene. The hypothesis of lower steady-state levels of toxic metabolic by-products is also supported by the observation that CR lowers the expression of cytochrome P450 isoforms IIIA and Cyp1b1 (involved in detoxification), Hsp105 (a heat shock factor), aldehyde dehydrogenase (an inducible enzyme in-

involved in detoxification of metabolic by-products) and an oxidative stress-induced protein of unknown function.

Reduction of endogenous damage by CR is apparently not limited to proteins, because CR reduced the expression of several DNA repair genes including XPE [a factor that recognizes multiple DNA adducts (Payne and Chu 1994)], RAD50 [involved in double-strand break repair (Petrini 1999)] and DNA polymerase beta [a DNA damage inducible polymerase (Fornace et al. 1989)]. Because skeletal muscle is largely a postmitotic tissue, a reduction in the expression of DNA repair genes is most consistent with a decrease in endogenous damage, as opposed to reduced mitotic activity. These observations are in agreement with the reduction in oxidative damage to DNA in calorie-restricted rodents (Sohal and Weindruch 1996), but contrast with the hypothesis that CR delays aging through an upregulation of DNA repair. How does CR reduce the generation of toxic by-products of metabolism? One putative mechanism of action is lowered metabolic rate due to reduced substrate availability for mitochondrial electron transport and subsequent ROS production, a view that is controversial based on observations of whole animal oxygen consumption measurements in CR rodents (Sohal and Weindruch 1996). We find molecular evidence to support a state of lower basal metabolic rate in CR mice through lowered expression of the thyroid hormone receptor alpha gene, a key mediator of metabolism (Fraichard et al. 1997).

Implications for aging and nutritional research

These data provided the first global assessment of a nutritional intervention at the molecular level and underscore the utility of high density oligonucleotide microarray gene expression analysis in the study of complex biological phenomena. Current estimates would suggest that the 6347 genes analyzed in this study represent in the range of 5–20% of the mouse genome (Pennisi 2000). The development of higher density gene arrays is now providing a more complete analysis of gene expression patterns of organismal senescence and the impact of specific nutritional interventions on this process. The observed collection of gene expression alterations in aging skeletal muscle is complex, reflecting the presence of myocyte, neuronal and vascular components. Additionally, changes in mRNA levels may not always result in a parallel alteration in protein levels; however, the complete or partial prevention of the majority of the observed aging alterations by CR suggests that gene expression patterns can be used to assess the biological age of the tissue under study. Extension of our study to other organs should result in the identification of hundreds of tissue-specific biomarkers of aging, facilitating the elucidation of aging mechanisms and the development of interventions. Importantly, the method described here is readily applicable to the investigation of aging in both nonhuman primates and humans through the use of small tissue biopsies (100 mg or less with current technology).

Because the pathophysiology of aging is complex, it is likely that a detailed analysis of gene expression patterns will reveal organ-specific alterations. This prediction is supported by our recently reported findings from two brain regions (neocortex, cerebellum) in this mouse model, which shared an age-associated up-regulation of stress response genes with the gastrocnemius but differed from muscle in showing increased expression of genes involved in the inflammatory response (Lee et al. 2000). Also, microarray analysis of senescence in replicative tissues (Ly et al. 2000) has revealed aging patterns differing markedly from those which we have observed in muscle and brain. Genetic analysis of replicative senescence in *Saccharo-*

TABLE 2

Global view of transcriptional changes in mouse gastrocnemius muscle induced by aging and caloric restriction

Aging	Caloric restriction
↑ Stress response Induction of: Heat shock response DNA damage inducible genes Oxidative stress inducible genes ↓ Energy metabolism Reduced glycolysis Mitochondrial dysfunction ↑ Neuronal injury Reinnervation Neurite extension and sprouting	↑ Protein metabolism Increased synthesis Increased turnover ↑ Energy metabolism Upregulation of glycolysis, gluconeogenesis, and the pentose phosphate shunt ↑ Biosynthesis Fatty acid synthesis Nucleotide precursors ↓ Macromolecular damage Suppression of: Inducible heat shock factors Inducible detoxification systems Inducible DNA repair systems

myces cerevisiae has been linked to the accumulation of extra-chromosomal rDNA gene circles (Sinclair and Guarente 1997), whereas replicative senescence of human cells in vitro is associated with telomere shortening (Bodnar et al. 1998). Nevertheless, the approach outlined here should allow the identification of common patterns of transcriptional alterations among postmitotic tissues, providing a framework for investigating nutritional and pharmacological interventions.

How does our analysis fit with the prevailing theories of aging and the postulated mechanisms of aging retardation by CR? A summary of global changes induced by aging and the contrasting effects of CR are shown in Table 2. The obvious transcriptional activation of stress response genes that play a role in the processing of damaged or misfolded proteins during aging and the prevention of this induction by CR suggest a central role for protein modifications in aging of skeletal muscle. In fact, aging is characterized by an exponential increase of oxidatively damaged proteins that is indicated by loss of sulfhydryl groups, carbonylation and loss of catalytic activity (Berlett and Stadtman 1997). Previous analyses of metabolic rates in CR animals have led to the suggestion that this life-extending regimen acts through a reduction in metabolic rate, resulting in a lower production of toxic by-products of metabolism (Sohal and Weindruch 1996). The CR-mediated reduction of mRNA encoding inducible genes involved in metabolic detoxification, DNA repair and the response to oxidative stress support this view, because it implies lower substrate availability for these systems. Additionally, our analysis strongly suggests that an equally important mechanism of action may be a metabolic shift toward increased biosynthesis and macromolecular turnover. A hormonal trigger for this shift may be an alteration in the insulin-signaling pathway, a finding that links our observations to those obtained through the genetic analysis of aging in the nematode *Caenorhabditis elegans*. A consistent theme that emerges from our study is the importance of highly conserved pathways, such as the heat shock response and DNA repair, in understanding the aging process. Therefore, continued research in model organisms

such as *Escherichia coli* and *Saccharomyces cerevisiae*, as well as multicellular organisms, will provide key insights to guide the interpretation of information obtained in mammals through DNA microarray technology.

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