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Tissue specific and non-specific changes in gene expression by aging and by early stage CR

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Abstract

Aging alters the expression of a variety of genes. Calorie restriction (CR), which extends life span in laboratory rodents, also changes gene expression. This study investigated changes in gene expression across 3 different tissues from the same mouse to examine how aging and early stage CR influence gene expression in different tissues of an organism. Expression profiling of heart, liver, and hypothalamus tissues was done in young (4–6 months) ad libitum fed (AL), young CR (2.5–4.5 months of CR), and old (26–28 months) AL male C57BL/6 mice. Aging significantly altered the expressions of 309, 1819, and 1085 genes in heart, liver, and hypothalamus tissues, respectively. In 9 genes, aging altered expression across all 3 tissues although the regulation directions did not agree across all 3 tissues for some genes. Early stage CR in young mice significantly changed the expressions of 192, 839, and 100 genes in heart, liver, and hypothalamus tissues, respectively, and 7 genes altered expression across all 3 tissues; 3 were up regulated and 4 were down regulated. The results of **Gene Ontology (GO)** Biological Process analysis indicated up regulation of antigen processing/presentation genes by aging and down regulation of stress response genes by early stage CR in all 3 tissues. **The comparison of the results of aging and short term CR studies showed there were 389 genes, 18 GO biological processes, and 20 GO molecular functions in common.**

Keywords

Gene expression; Microarray; Aging; Calorie restriction

1. Introduction

With the sequencing of genomes and the advent of high-density array technology, gene expression arrays such as cDNA or oligonucleotide microarrays have emerged as a powerful tool to measure genome-wide gene expression in cells and tissues. In comparison with more traditional methods of analysis, microarrays have been compared to “turning on a light after trying to discern the details of one’s surroundings with a torch” (Editorial, 1998). Gene expression arrays have been used to measure gene expression profiles across multiple species from yeast to human, and across many treatment conditions from cancer to starvation. Since gene expression arrays allow rapid screening and quantification of differences in large groups of functionally related genes, this technology is well suited to systematically study the complex, multigenic processes induced by aging and by calorie restriction (CR).

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Although new genetic and pharmacologic interventions that extend mammalian life span are emerging (Amstrong et al., 1991; Carrillo et al., 1994; Freisieben et al., 1994; Miller, 1999), CR remains the only intervention repeatedly shown to increase life span and delay a wide spectrum of age-related diseases and physiological changes in laboratory rodents (Masoro, 1988, 2000). Restriction of total energy, not reduced protein or fat energy, appears to underlie the major contribution of reduced food intake to extend life (Masoro, 1992). The greatest extension of life span is achieved when CR is initiated at early ages and sustained throughout life, although the benefits of CR are observed when CR is initiated in middle age and sustained throughout life (Yu et al., 1985). These data indicate that the anticipating effects of CR begin shortly after its onset and are cumulative.

Altered gene expression undoubtedly plays a significant role in directing aging effects and the anti-aging actions of CR. Although there have been many studies of altered gene expression by aging and/or by CR in a single tissue using gene expression arrays, no studies on multiple tissues from the same animal have been reported. It is speculated that some genes may alter in a tissue specific manner and others may alter universally across all tissues. Uncovering the pattern of altered gene expression by aging and/or CR in an organism as a whole should shed more light on the aging process and on tissue-wide anti-aging mechanisms of CR. To this end, we investigated the gene expression profiles induced by aging and by early stage CR across 3 tissues from the same animal. Using Affymetrix GeneChips® with approximately 12000 genes, we measured the global gene expression in the heart, liver, and hypothalamus of young (4–6 months of age), old (26–28 months of age), and young CR (4–6 months of age with 2.5–4.5 months of CR) mice.

2. Materials and methods

2.1. Animals

Parental C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in the Animal Core of the Nathan Shock Center at the University of Texas Health Science at San Antonio. All mice consumed ad libitum (AL) Harlan Teklad LM-485 mouse/rat sterilizable diet 7912 (Madison, WI) until 6 weeks of age. At 6 weeks, AL group mice were allowed to continue on this diet until killed (young and old groups). The CR group mice were restricted to 60% of the mean food intake of the AL group until killed (young CR group). The mice went to CR immediately from the AL diet and this protocol has been used by the University of Texas Health Science at San Antonio group for more than 25 years. Extensive pathological, behavioral, and functional tests throughout this time have revealed no developmental repercussions from this protocol. CR mice were given their food allotment 1 h before the start of the dark phase of the light cycle. Mice were kept on a 12: 12 h light-dark cycle (lights on at 06:00 h).

Sentinel mice were tested monthly by a veterinary pathologist in Laboratory Animal Resources at the University of Texas Health Science Center at San Antonio. Every 6 months, the presence of murine virus antibodies was monitored with serum samples from sentinel mice by BioReliance Co. (Rockville, MD). All tests were negative. All procedures involving the use of mice were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center and the Subcommittee for Animal Studies at the Audie L. Murphy Memorial Veterans Hospital.

2.2. Tissue collection and RNA preparation

Heart, liver, and hypothalamus tissues from 18 young (4–6 months of age), 18 young CR (4–6 months of age; 2.5–4.5 months of CR), and 18 old male mice (26–28 months of age) were collected between 10:00 and 12:00 h. The tissues were quickly frozen in liquid nitrogen and

stored at −80°C until RNA extraction. Total RNA was extracted from each tissue as previously described (Sambrook et al., 1989). The RNA yield of each sample was determined spectrophotometrically, assuming 1 optical density at 260 nm (OD_{260}) unit = 40 mg/L (Sambrook et al., 1989). The quality of total RNA extracted from each sample was monitored by A260:A280 ratio and 1.0% agarose formaldehyde gel electrophoresis. All samples had 260:280 ratios of ~2 and exhibited discrete 28s and 18s bands. Several samples were randomly chosen and subjected to Northern Blot analysis for further mRNA quality control using glyceraldehydes-3-phosphate dehydrogenase as a probe (data not shown).

2.3. Screening of mRNA by Affymetrix GeneChip® arrays

Heart, liver, and hypothalamus tissues from 18 each of young, young CR, and old mice (15 out of 18 mice were used for the old heart group) were used. RNAs from 3 mice were pooled together (the same amount of RNA from each mouse) to generate 6 pooled samples (although 6 pooled samples were available, only 5 pooled samples were hybridized for the old heart group) for each treatment group [i.e., Heart Young 1–6 (n=6), Heart Young CR 1–6 (n=6), Heart Old 1–5 (n=5), Liver Young 1–6 (n=6), Liver Young CR 1–6 (n=6), Liver Old 1–6 (n=6), Hypothalamus Young 1–6 (n=6), Hypothalamus Young CR 1–6 (n=6), and Hypothalamus Old 1–6 (n=6) GeneChip® arrays]. We used Murine Genome U74A Version 2 GeneChips® containing oligonucleotide probes for approximately 12,000 genes from Affymetrix (Santa Clara, CA). Prior to the labeling reaction, RNA samples were subjected to a cleanup process using columns from RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA). We followed the vendor's protocols for GeneChip® hybridization and scanning (Han et al., 2004a).

2.4. Statistical analysis of microarray data

Affymetrix GeneChip® Operating Software (GCOS version 1.1.1, Affymetrix, Santa Clara, CA) was used to quantitate each GeneChip®. The summary intensities for each probe (as contained in the CEL files) were read into DNA-Chip Analyzer (dChip) (Li and Wong, 2001), version 1.3 and GeneChip® normalization and standardization was carried out as described previously (Fu et al., 2004).

In order to determine which genes differed significantly in expression between the comparison groups, we ran unpaired t-tests, a commonly used method to evaluate the differences in means between two groups. The t-test comparison assumes the data are approximately normally distributed, and the variances of the separate groups are approximately equal. For this reason, we standardized and log transformed the data prior to analysis. For each tissue, we ran two unpaired t-test comparisons to evaluate the differences in means between groups: 1) Young versus (v) Old and 2) Young AL (data from Young was also used as Young AL) v Young CR. In order to correct for multiple testing, we calculated the Hochberg and Benjamini (Hochberg and Benjamini, 1990) false discovery rate (FDR) and set the FDR adjusted p-value (α) for the unpaired t-test results at less than 0.01 for Young v Old comparisons and 0.05 for Young AL v Young CR comparisons. The results were further restricted by deleting those probe sets with "absent" GCOS detection calls across all chips in both comparison groups. Considering the Gene Bank Accession number to represent unique genes, we deleted repeated accession numbers except in cases when the probe set name designation indicated that the probe sets recognized alternative transcripts from the same gene. Otherwise, we discarded the repeated accession number results for those probe sets that were not unique to a single gene (AffyMetrix, inc., 2004).

We used the Expression Analysis Systematic Explorer (EASE**; Hosack et al., 2003)** to statistically test for significant coregulation between our results and the Gene Ontology (GO) Consortium categories, Biological Process **and Molecular Function. At the time of writing, there were 11,322 annotated biological process and 7,460 molecular function**

categories. Instead of ranking functional clusters by the number of selected genes per category, this software ranked functional clusters by statistical over-representation of individual genes in specific categories relative to all genes in the same category. The EASE score attenuates the significance of categories carried by a few genes and slightly penalizes categories supported by many genes in order to yield more robust findings. The EASE score can be thought of as analogous to a p-value when deciding cut-offs for significance levels and indicates the probability of finding the number of selected genes per category by chance alone. We set the EASE score less than 0.05 to identify significant biological process **and molecular function** categories. **To estimate the global FDR for biological process and molecular function categories, the statistical option within the EASE program was used.**

In order to test for the significance of the overlap between tissues and the overlap between selected genes and EASE results we utilized a Pearson's chi-square test (χ^2) (Chernoff and Lehmann, 1954) and a hypergeometric analysis (Bateman and Erdélyi, 1953), respectively. A Pearson's chi-square tests a null hypothesis that the relative frequencies of occurrence of observed events follow a specified frequency distribution. In probability theory and statistics, the hypergeometric distribution is a discrete probability distribution that describes the number of successes in a sequence of *n* draws from a finite population without replacement.

2.5. Real time quantitative reverse transcription polymerase chain reaction (QRT-PCR)

The same sources of RNA used for the GeneChip® array study were used for the real time QRT-PCR. Primers were designed using the OligoPerfect™ Designer (Invitrogen, Carlsbad, CA) and purchased from Invitrogen (see supplemental Table 1 at http://www.sciencedirect.com for primer sequences and their annealing temperatures). The 18S rRNA was used as an internal control for PCR quantitation. PCR reactions were carried out as previously described (Fu et al., 2004). Relative quantitation of gene expression was performed using the method published previously (Cook et al., 2004).

3. Results

3.1. Identification of altered gene expression by aging and functional clustering of genes

Altered gene expression by aging was measured using the heart, liver and hypothalamus tissues and Affymetrix oligonucleotide arrays. The results generated from the comparisons of young and old by tissue were summarized in Table 1. The numbers of genes up regulated and down regulated by aging were indicated as well as the total numbers of genes identified [consult supplemental Table 2 (http://www.sciencedirect.com) for the complete list of genes]. Nine genes significantly altered expression by aging across all 3 tissues (Table 2). Amylase I, leucine rich protein B7 gene, and an unknown gene were up regulated in all 3 tissues while RIKEN cDNA 1500005K14 gene was down regulated in all 3 tissues. Three other genes (P53-variant, paired box gene 6, and vascular cell adhesion molecule 1) were up regulated in heart and liver tissues, but down regulated in the hypothalamus. Table 3 listed the numbers of altered genes in common across each combination of 2 tissues (supplemental Table 3 at http://www.sciencedirect.com/ contains the complete list of genes). For example, there were 56 genes up regulated in hearts which were also altered in one of either liver or hypothalamus tissues by aging. Twenty-eight and 12 of them were up regulated in liver and hypothalamus tissues, respectively, and 4 and 12 of them were down regulated in liver and hypothalamus tissues, respectively. Aging down regulated 35 genes in the heart which were also altered in one of either liver or hypothalamus tissues. Among them, 18 and 3 genes were down regulated in liver and hypothalamus tissues, respectively, and 8 and 6 genes were up regulated in liver and hypothalamus tissues, respectively.

Using the EASE analysis tool, we identified **100** biological process (EASE score less than 0.05 **and global FDR less than 8%**) **and 92 molecular function** categories (EASE score less than 0.05 **and global FDR less than 14%**) in which genes influenced by aging were overrepresented in 3 tissues (see supplemental Table 4 at http://www.sciencedirect.com). Because many genes have more than one function (pleiotropic), some of the identified genes were included in more than one category. Table 4 listed the common representative categories for age related up or down regulated genes in 2 or more tissues. **In biological processes,** antigen processing/ presentation category was significantly up regulated in all 3 tissues. Immune response category was significantly up regulated in heart and liver but not in the hypothalamus, whereas biosynthesis category, especially macromolecule biosynthesis category, was significantly down regulated by aging in heart and liver but not in the hypothalamus. Ion transport category, particularly metal ion transport category, was significantly down regulated in heart and hypothalamus, and ribosome biogenesis and assembly category was significantly up regulated in liver and hypothalamus. Cell growth and lipid metabolism categories were significantly up regulated in the liver, but were significantly down regulated in the hypothalamus. **In molecular functions, MHC class I receptor activity was up regulated in all 3 tissues. Oxidoreductase activity was up regulated in heart and liver but not in hypothalamus while transferase activity was down regulated in liver and hypothalamus but not in heart. The regulation directions of other identified molecular functions either did not agree in 2 tissues or agreed in 2 tissues but not in all 3 tissues and, in liver, 4 molecular functions were both up and down regulated.**

A hypergeometric analysis of the biological process categories for each set of two tissues suggested a global correlation in age-regulation between the heart and liver and the heart and hypothalamus ($p<0.05$ and $p<0.01$ respectively). Similarly, the heart and liver showed a global correlation in age-regulation of the molecular function categories ($p<0.01$). It appears that for some pairs of tissues, the age regulated biological process or molecular function categories from one tissue are more overlapped with the age regulated biological process or molecular function categories found in another tissue.

3.2. Identification of altered gene expression in early stage calorie restriction and functional clustering of genes

Since the anticipating effects of CR begin even shortly after its onset, we measured CR effects on gene expression in early stage CR in young mice. Heart, liver, and hypothalamus tissues from the same animal were used. The numbers of genes identified as significantly differentially expressed in the comparison of young AL to young CR mice in each tissue were listed in Table 5. The numbers of genes up regulated and down regulated by CR were indicated as well as the total numbers of genes identified (see supplemental Table 5 at http://www.sciencedirect.com for gene names, gene bank accession numbers, fold changes, and p-values). Seven genes significantly altered expression by CR across all 3 tissues (Table 6). Three genes among them were up regulated, and the remaining 4 genes were down regulated in all 3 tissues. The 3 up regulated genes were period homolog 2 (Drosophila), RNA binding motif protein 3, and transmembrane 4 superfamily member 7; the 4 down regulated genes were heat shock protein (105 kDa), protein disulfide isomerase-related protein, CD8 antigen β chain, and CDK2-associated protein 2. Table 7 listed numbers of altered genes in common across each combination of 2 tissues (supplemental Table 6 at http://www.sciencedirect.com/ contains complete list of genes). For example, there were 18 genes up regulated by CR in the heart that were also altered in either liver or hypothalamus tissues. Sixteen and 1 of them were up regulated in liver and hypothalamus tissues, respectively, and 1 was down regulated in the liver. CR down regulated 20 genes in the heart that were also altered in either liver or hypothalamus tissues. Among them, 5 and 10 genes were down regulated in liver and hypothalamus tissues, respectively, and 5 genes were up regulated in the liver.

Using the EASE analysis tool, we identified **68** biological process **and 51 molecular function** categories **(EASE score less than 0.05 and global FDR less than 20% for both biological process and molecular function)** in which genes influenced by CR were overrepresented (see supplemental Table 7 at http://www.sciencedirect.com). Again, because many genes are pleiotropic, some of the identified genes appeared in more than one category. Table 8 listed the shared representative categories for genes up or down regulated by CR in 2 or more tissues. **In biological processes**, stress response genes were significantly down regulated by CR in all 3 tissues. Responses to abiotic stimulus, external stimulus, and heat categories were significantly down regulated in heart and hypothalamus but not in the liver, whereas response to pest/pathogen/parasite category was significantly down regulated in heart and liver but not in the hypothalamus. Carbohydrate metabolism category was significantly down regulated in the heart, but was significantly up regulated in the liver, and protein folding category was significantly down regulated by CR in liver and hypothalamus. **In molecular functions, chaperone activity, including heat shock protein activity, was up regulated in all 3 tissues. Hydrolase and isomerase activities were down regulated in heart while hydrolase activity was up regulated and isomerase activity was both up and down regulated in liver.**

In order to investigate whether there is a global overlap in CR-regulation of biological process or molecular function categories between each set of two tissues, we ran a hypergeometric analysis. The results indicated a global correlation in CR-regulation of molecular function categories exists between the liver and hypothalamus ($p<0.05$). In other words, the CR regulated molecular function categories from the liver are enriched with the CR regulated molecular function categories found in the hypothalamus.

3.3. Gene expressions and biological processes that altered by aging and CR

There were 389 genes that were identified as significantly differentially expressed not only in the comparison of young v old but also in the comparison of young AL v young CR (supplemental Table 8 at http://www.sciencedirect.com/ contains complete list of genes). All 389 genes showed the commonality in a single tissue (i.e., one tissue from young v old comparison and one tissue from AL and CR comparison) except 2 genes. One was RIKEN cDNA 1500005K14 gene. While the expression was decreased by aging in all 3 tissues [fold change (FC) in heart -1.34, liver −1.21, and hypothalamus −1.44], by CR it was decreased in the heart (FC −1.72) and increased in the liver (FC 1.21). The other was a heat shock protein (105 kDa) gene. Aging decreased the expression in the heart (FC −1.79) and the liver (FC −1.86), whereas CR decreased the expression in all 3 tissues (FC in heart −2.11, liver −2.39, and hypothalamus −1.45).

When we compared 68 significantly over represented GO biological processes from young AL v young CR comparison with the 100 categories from the young v old comparison, there were 18 categories in common (Table 9). Antigen processing/presentation category was increased by aging in all 3 tissues and it was decreased by CR in the heart. Immune response category was up regulated by aging in heart and liver and down regulated by CR in the heart. Aging increased the response to stress category in the heart while CR reduced this category in all 3 tissues. Responses to external stimulus, pest/pathogen/parasite, and biotic stimulus and defense response categories were all increased by aging but decreased by CR in the heart. **We also compared 92 significantly over represented GO molecular function categories from young AL v young CR comparison with the 51 categories from the young v old comparison. There were 20 categories in common (**Table 9).

3.4. Validation of selected GeneChip® array results by real time QRT-PCR

To validate the microarray data, real time QRT-PCR was carried out on 31 randomly chosen genes. Some of the genes were tested in more than one tissue and/or in both treatments (i.e., young v old and AL v CR). Fifty five array and QRT-PCR ratio (FC) pairs were generated. Some of the tested array ratios were from significantly differentially expressed genes, others were not. The fold changes detected by array were similar to those by real time QRT-PCR in most comparisons. Table 10 listed 25 out of 55 pairs. In Table 10, the array and the real time QRT-PCR results were given as point estimates of the ratios of the two comparison groups and the 95% confidence intervals were indicated in parentheses. When the 95% confidence intervals for the QRT-PCR ratios overlapped with the corresponding 95% confidence intervals for the array ratios, the array ratios were considered validated. Indeed, the results confirmed that 48 out of 55 array and QRT-PCR ratio pairs were equivalent, as indicated by the fact that their 95% confidence intervals overlapped. Five of the 7 invalidated ratios resulted from no expression detection by PCR in one of the 2 comparison groups (either young or old sample and either AL or CR sample). These genes were considered to be either turned-on or turned-off by aging or by CR.

4. Discussion

While there have been many studies on gene expression in a single tissue from an animal, to date there has been no study that reported gene expressions across multiple tissues from the same animal. Furthermore, relatively little information exists on the initial response to CR in young animals even though such information could be valuable in probing the mechanisms that ultimately give rise to the anti-aging phenotype. For that reason, using Affymetrix GeneChips®, we investigated the effect of old age on gene expression in the heart, liver, and hypothalamus and the effects of CR shortly after its initiation on gene expression in these tissues in young mice. The 3 tissues chosen are those which play major roles in maintenance of homeostasis within the body and/or are known to be altered by both aging and nutrition (Masoro, 1992).

Aging changed the expression of 309, 1819, and 1085 genes, in heart, liver, and hypothalamus tissues, respectively (Table 1). There were 9 genes that changed expression across all 3 tissues (Table 2). **Out of the remaining 300 genes that altered expression by aging in the heart, 91 genes also changed expression in either liver or hypothalamus tissues, 231 genes that altered in the liver also changed in either heart or hypothalamus tissues, and 206 genes that changed in the hypothalamus also changed in either heart or liver tissues (**Table 3). Thirty-two percent (100/309), 13% (240/1819), and 20% (215/1085) of the genes that changed expression by aging in heart, liver, and hypothalamus tissues, respectively, also altered expression in another tissue (or tissues **; χ^2=76.4, df=2, p<0.001**). In other words, 68%, 87%, and 80% of altered genes were specific to the heart, liver, or hypothalamus, respectively. Of course, these genes may alter expression in tissues other than in the 3 we tested. However, our results indicated that the liver was the tissue whose gene expression changed the most by aging. Moreover, of the 3 tissues, the liver showed the highest percentage of tissue specific results.

In order to investigate whether there is a global overlap in age-regulation between each set of two tissues, we ran a hypergeometric analysis. The results indicated a global correlation in age-regulation exists between each set of two tissues (p values ranged from $p<0.05$ to $p<0.0001$ for each regulation by set combination). In other words, we see more overlap between two tissue sets of age regulated genes than we would expect by chance alone.

RIKEN cDNA 1500005K14 gene (cfm; AW047875) was the only gene down regulated by aging in all 3 tissues. Cfm is a novel gene that does not have any known functional domains, but is conserved in several different species and has a site of phosphorylation by MAP kinase

in one of the conserved domains (Hirano et al., 2005). Amylase 1, salivary (amy1; J00356) was one of the up regulated genes in all 3 tissues. Mouse amy1 encodes alpha-amylase 1 enzyme that is mainly produced in parotid and liver tissues (Hagenbuchle et al., 1980). The enzyme catalyzes the cleavage of alpha-1, 4-glucosidic linkages between glucose molecules in starch, and thus plays an important role in starch digestion (Mielenz, 1983). In the parotid, aging was associated with a decrease in amylase release and reduction in salivary function (Liu et al., 2001; Mahay et al., 2004; Nagler and Hershkovich, 2005). It was unexpected to see the up regulation of amy1 by aging in the heart, liver and hypothalamus tissues, but all samples indicated the increased expression of the gene by aging (and all of the chips' Microarray Suite version 5.0 detection calls were "present"). Perhaps the induction of amy1 was a compensatory action for the decreased parotid function and reduced digestive activity of old individuals. Another gene of interest was vascular cell adhesion molecule 1 (vcam1; U12884) which was up regulated in heart and liver, but down regulated in the hypothalamus. Vcam1, a member of the immunoglobulin superfamily, mediates leukocyte-endothelial cell adhesion and signal transduction, and may play an important role in the regulation of inflammation in various vascular disorders (Meager, 1999; Merat et al., 2000; Cook-Mills et al., 2004). Previously, the age related up regulation of vcam1 in mouse aortas was reported, and it paralleled the increased incidence of arthrosclerosis (Alexander, 1998; Merat et al., 2000; Zou et al., 2006). It has been well documented that age related induction of vcam1 results from increased oxidative stress, and that over expressed vcam1 in turn recruits excessive leukocytes that trigger a prolonged inflammatory response and age related vascular diseases (Yu and Chung, 2001a; Yang et al., 2004; Zou et al., 2006). Therefore, the increased expression of vcam1 in aged liver and heart may indicate a higher oxidative stress level and provide evidence to explain the higher artherosclerosis rate among aged individuals. The reason for the down regulation of this gene in the aged hypothalamus is not clear since the aged hypothalamus has been reported to be in a pro-inflammatory state (Prolla, 2002). However, in the aged hypothalamus, we found that the complement component genes (C1qa; X58861, C1qb; M22531, C1qc; X66295, and C4; 06454) were significantly up regulated (see supplemental Table 2 at http://www.sciencedirect.com). Evidently, different tissues used different genes to regulate the same biological process.

According to the results from the functional grouping by biological processes, one of the notable alterations by aging was the up regulation of immune system related genes. For instance, genes involved in immune response were up regulated in heart and liver tissues, and genes involved in antigen processing/presentation were up regulated in all 3 tissues. Similar results also have been observed in mouse hippocampus (Terao et al., 2002), rat hippocampus (Blalock et al., 2003), and rhesus monkey skeletal muscle (Kayo et al., 2001) tissues. Taken together, the up regulation of immune related genes in aging does not appear to be tissue specific; to the contrary, in fact, it seems to be universal. This would suggest an increased risk for infection and tumors in aged animals. In contrast with this observation, there is a functional decrease observed with age in the peripheral immune response (Miller, 1996; Ponnappan, 1998; Terao et al., 2002; Renshaw et al., 2002). The basis of this dichotomy remains unclear at present (Terao et al., 2002). We speculate that the increased expression of immune response genes in aged animals may be a compensatory response to the age dependent decline in immune function. For example, the increased expression of MHC class I and MHC class II genes may be in compensation for the declined function of antigen presenting cells (Effros and Walford, 1984; Giardina and Hubbard, 2002; Plowden et al., 2004a, 2004b), while the increased expression of immunoglobulin related genes may be in compensation for the reduced ability of B cells to produce high affinity antibodies (Lord et al., 2001; Han et al., 2004b).

In the heart, liver, and hypothalamus, 192, 839, and 100 genes changed expression in early stage CR, respectively (Table 5). Seven genes changed expression in all 3 tissues (Table 6). There were 38 genes altered in the heart that also changed expression in either liver or

hypothalamus tissues; 36 genes altered in the liver also changed in either heart or hypothalamus tissues; and 20 genes altered in the hypothalamus also changed in either heart or liver tissues (Table 7). Twenty-three percent (45/192), 5% (43/839), and 27% (27/100) of the genes changed in heart, liver, and hypothalamus tissues, respectively, were also altered in another tissue (or tissues **; χ^2=90.1, df=2, p<0.001**). Conversely, 77%, 95%, and 73% of the genes with altered expressions in heart, liver, and hypothalamus tissues, respectively, were altered only in the single tissue. Of course, it is possible that these genes alter expression in tissues other than in the 3 tissues we tested. As with aging, the liver changed gene expression the most in early stage CR and shared the least number of genes with the other tissues.

A hypergeometric analysis by regulation direction of the genes for each set of two tissues suggested a global correlation in CR-regulation is present for the heart and liver up regulated set and the liver and hypothalamus down regulated set ($p<0.01$ for each set). It appears that in some cases the CR regulated genes from one tissue are enriched with the CR regulated genes found in another tissue, but not as pervasively as found in the aging results.

One of the noteworthy CR effects from the analyses of 3 tissues was the down regulation of heat shock protein genes (hsps). For example, hsp110 (105 kDa; L40406) was down regulated in all 3 tissues (Table 6); hsp84 (M18186) and Dnajb10 (AI843164) were down regulated in liver and hypothalamus tissues; hsp47 (X60676) was down regulated in heart and hypothalamus tissues; hsp86 (J04633) was down regulated in heart and liver tissues; hsp4 (AA615831) was down regulated in liver tissues; and hsp70-1 (AF109906), hsp70-3 (M12571), hsp70-5 (AJ002387), Dnajb1 (AB028272), Dnajb11 (AW122551), and Dnajc3 (U28423) were down regulated in hypothalamus tissues (see supplemental Table 5 at http://www.sciencedirect.com). Hear shock proteins (Hsps) are cytoprotective proteins that act as essential intracellular chaperones by helping in the refolding of misfolded proteins and assisting in their elimination (Wang et al., 2003;Thomas et al., 2005). Hsp 110 is a major Hsp in mammals and eukaryotic cells in general (Wang et al., 2003). Unlike other hsps, hsp110 has been cloned and studied only within the last few years. One important property of Hsp110 is its ability to bind and chaperone with high efficiency during heat shock (Wang et al., 2003). Hsp70, one of the most prominent Hsps, plays a critical role in protecting cells against the adverse effects of hypothermia (Heydari et al., 1993;Liu et al., 2006). The production of Hsps is thought to be a major endogenous cellular defense mechanism in inflammation and aging (Yu and Chung, 2001b). Thus, the down regulation of many hsps, as observed by us and by other researchers (e.g., Cao et al., 2001;Weindruch et al., 2001), may suggest a lowered pro-inflammatory state.

The most notable biological process category for genes influenced by CR was stress response which was significantly down regulated in all 3 tissues. Similar results have been observed in muscle, liver, neocortex, and cerebellum (Cao et al., 2001). Increased expression of stress resistance genes seems to be a common anti-aging effect of CR across different tissues. Furthermore, Cao and colleagues (Cao et al., 2001) revealed that although long term CR (27 months of age; CR started after weaning) led to a significant increase of mRNA expression for stress resistance genes, this action actually emerged in the early stage of CR (4 weeks of CR in 34 month old mice). Taken together, these results suggest that stress resistance is an initial and a long lasting response to CR and may rapidly and persistently result in the beneficial effect of withstanding both internal and external insults. This finding also provides evidence to support the hypothesis that the ability of CR to increase stress resistance is at the core of its anti-aging effects (Chung et al., 2001).

When we examined the comparative effects of aging and early stage CR on gene expression profiles (Table 9), we noticed that antigen processing/presentation and immune response categories were significantly up regulated by aging in more than one tissue (antigen processing/

presentation all 3 tissues, immune response heart and liver tissues), but the effect was significantly down regulated by early stage CR only in the heart. Also, many stimulus response related genes (stress, external stimulus, pest/pathogen/parasite, and biotic stimulus, and defense response) were significantly up regulated by aging and significantly down regulated by early stage CR in the heart. It seemed that the heart is the most protected among the 3 tissues from infection and insults in early stage CR. Unfortunately, since we did not have long term CR animals from the same cohort of mice, we could not examine the long term effects of CR across 3 tissues.

A particularly interesting finding from our analyses across 3 tissues was that, in most cases, the precise genes involved in any given biological process differed in the aging versus the CR comparisons. In other words, genes involved in a biological process up regulated by age were not the same as those involved in the identical biological process down regulated by CR, for instance. Apparently, early stage CR reduced age dependent changes using different genes than those that altered expression during aging, yet all were within the same biological function. Likewise, genes involved in the same function were not the same across different tissues in most instances.

We compared genes we identified in our study with genes identified as differentially expressed by CR and/or aging in heart (Bodyak et al, 2002; Bronikowski et al., 2003; Dhahbi et al., 2006; Edwards et al., 2003; Lee et al., 2002), liver (Cao et al., 2001; Dhahbi et al., 2004; Tsuchiya et al., 2004), and hypothalamus (Jiang et al., 2001) tissues of mice from previously published gene expression array studies. Supplemental Table 9 (at http://www.sciencedirect.com/) showed the result of the detailed comparison. In heart, 5 (2 up and 3 down regulated) and 37 genes (10 up and 27 down regulated) that we identified for their differential expression by aging and by CR, respectively, were also identified by others. There were 7 (3 up and 4 down regulated) and 40 genes (29 up and 11 down regulated) that altered their expression by aging and by CR, respectively, in common between our study and other studies in liver. In hypothalamus, only 4 genes (2 up and 2 down regulated) that altered their expression by aging showed matching results with the other study. Presumably the discrepancy came from the use of different strain and/or age of animals and/or CR regimen in their study versus our study.

In summary, we identified differentially expressed genes in the heart, liver, and hypothalamus tissues of mice by aging and/or by short term CR (2.5–4.5 months of CR). Additionally, the genes identified as differentially expressed were analyzed for over represented functional clusters using the EASE bioinformatics software package. To confirm our microarray results, we carried out real time QRT-PCR and generated 55 array and QRT-PCR ratio (FC) pairs. Excellent overall agreement was demonstrated between these two methods. Aging affected gene expression more significantly and broadly than did CR in all 3 tissues and there were tissue specific gene expression profiles in responses to aging and to CR. Whether the changes in gene expression identified in this study resulted in corresponding changes in protein expression/activity is not known. Nevertheless, this study uncovered genes affected by aging and by short term CR across 3 different tissues within an animal. Hopefully, these findings will inform investigators with expertise on the specific genes, allowing them to pursue more mechanistic studies to reveal the physiological significance of these changes in the anti-aging actions of CR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

The numbers of genes identified as statistically significantly differentially expressed in the comparison of young versus old mice in the heart, liver, and hypothalamus[a].

Tissue	Unique GenBank accession no. (n)	Up regulated (n)	Down regulated (n)
Heart	309	150	159
Liver	1819	828	991
Hypothalamus	1085	460	625

[a] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.01.

Table 2

Genes identified for altered expression in common across all three tissues in the young versus old comparison[a].

Gene name	GenBank accession no.	Heart FC[b]	Liver FC	Hypothalamus FC
amylase 1, salivary	J00356	2.14	1.72	1.69
Unknown, Image 778130	AA419684	1.35	1.25	1.27
leucine rich protein, B7 gene	AV257486	1.25	1.29	1.24
P53-variant (p53)	U59758	1.48	1.27	−1.11
paired box gene 6	X63963	2.22	1.30	−1.37
vascular cell adhesion molecule 1	U12884	1.84	3.04	−1.39
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily f, member 1	AA832935	−1.13	1.15	1.20
centaurin, gamma 2	AW123016	−1.28	−1.18	1.31
RIKEN cDNA 1500005K14 gene	AW047875	−1.34	−1.21	−1.44

[a] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.01.

[b] Positive fold changes (FC) are up regulated by age (old/young) and negative FC are down regulated by age (young/old), computed by dChip using the PM-only model-based expression index values calculated on normalized arrays.

Table 3

Numbers of genes identified for altered expression in common across each combination of two tissues in the young versus old comparison[a].

Heart up regulated (n)	Heart down regulated (n)	Liver up regulated (n)	Liver down regulated (n)	Hypothalamus up regulated (n)	Hypothalamus down regulated (n)
28		28			
4			4		
	18		18		
	8	8			
12				12	
12					12
	3				3
	6			6	
		53		53	
		35			35
			60		60
			25	25	

[a]Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.01.

Table 4

Significantly over represented Gene Ontology Biological Processes and Molecular Functions[a] in common across tissues in the young versus old comparison results[b].

Biological process	Heart (n)	Liver (n)	Hypothalamus (n)
antigen processing/presentation	5↑[c]	7↑	6↑
immune response	1 8↑	45↑	
biosynthesis	1 9↓[d]	82↓	
macromolecule biosynthesis	1 6↓	70↓	
ion transport	11↓		29↓
metal ion transport	8↓		20↓
ribosome biogenesis and assembly		1 2↑	1 0↑
lipid metabolism		45↑	29↓
cell growth		1 0↑	9↓
Molecular function			
hydrolase activity	23↑	94↑	28↓
metal ion binding	1 6↓	58↑	43↓
MHC class I receptor activity	3↑	4↑	6↑
nucleic acid binding	31↓	1 31↑	66↑
protein binding	25↓	111↑ ; 1 09↓	87↓
RNA binding	1 0↓	36↑ ; 32↓	25↑
binding	68↓	322↑ ; 367↓	
catalytic activity	52↑	204↑ ; 235↓	
intramolecular isomerase activity	3↑	6↓	
oxidoreductase activity	1 2↑	4↑	
blood coagulation factor activity		6↑	4↓
complement activity		6↑	5↑
lipid binding		1 2↑	1 3↓
phosphotransferase activity		38↓	4↑
structural molecule activity		42↓	38↑
transferase activity		83↓	54↓

[a] Significant biological process and molecular function categories are those with an EASE score less than 0.05.

[b] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.01.

[c] Up regulation

[d] Down regulation

Table 5

The numbers of genes identified as statistically significantly differentially expressed in the comparison of AL versus CR mice in the heart, liver, and hypothalamus[a].

Tissue	Unique GenBank accession no. (n)	Up regulated (n)	Down regulated (n)
Heart	192	78	114
Liver	839	550	289
Hypothalamus	100	22	78

[a] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.05.

Table 6

Genes identified for altered expression in common across all three tissues in the young AL versus young CR comparison results[a].

Gene name	GenBank accession no.	Heart FC[b]	Liver FC	Hypothalamus FC
period homolog 2 (Drosophila)	AF036893	2.96	2.49	1.38
RNA binding motif protein 3	AB016424	2.37	2.85	2.04
transmembrane 4 superfamily member 7	AW124470	2.08	1.41	1.23
heat shock protein, 105 kDa	L40406	−2.11	−2.39	−1.45
protein disulfide isomerase-related protein	AI842377	−1.36	−1.94	−1.31
CD8 antigen, beta chain	X07698	−1.33	−1.85	−1.36
CDK2-associated protein 2	AI835912	−1.16	−1.52	−1.12

[a] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.05.

[b] Positive fold changes (FC) are up regulated by CR (CR/AL) and negative FC are down regulated by CR (AL/CR), computed by dChip using the PM-only model-based expression index values calculated on normalized arrays.

Table 7

Numbers of genes identified for altered expression in common across each combination of two tissues in the young AL versus young CR comparison results[a].

Heart up regulated (n)	Heart down regulated (n)	Liver up regulated (n)	Liver down regulated (n)	Hypothalamus up regulated (n)	Hypothalamus down regulated (n)
16		16			
1			1		
	5		5		
	5	5			
1				1	
0					0
	10				10
	0			0	
		1		1	
		3			3
			5		5
			0	0	

[a]Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.05.

Table 8

Significantly over represented Gene Ontology Biological Processes and Molecular Functions[a] in common across tissues in the young AL versus young CR comparison results[b].

Biological process	Heart (n)	Liver (n)	Hypothalamus (n)
response to stress	14↓[c]	22↓	11↓
response to abiotic stimulus	11↓		9↓
response to external stimulus	31↓		14↓
response to heat	4↓		6↓
response to pest/pathogen/parasite	9↓	13↓	
carbohydrate metabolism	9↓	23↑[d]	
protein folding		8↓	6↓
Molecular function			
chaperone activity	8↓	9↓	10↓
heat shock protein activity	5↓	4↓	7↓
hydrolase activity	5↓	23↑	
isomerase activity	4↓	9↑ ; 7↓	

[a] Significant biological process and molecular function categories are those with an EASE score less than 0.05.

[b] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.05.

[c] Down regulation

[d] Up regulation

Table 9

Significantly over represented Gene Ontology Biological Processes and Molecular Functions[a] in common across tissues and across comparisons[b].

	Young v Old			Young AL v Young CR		
Biological process	**Heart (n)**	**Liver (n)**	**Hypothalamus (n)**	**Heart (n)**	**Liver (n)**	**Hypothalamus (n)**
antigen processing/presentation	5↑[c]	7↑	6↑	3↓[d]		
response to stress	1 7↑			1 4↓	22↓	11↓
immune response	1 8↑	45↑		1 9↓		
biosynthesis	1 9↓	82↓			53↑	
macromolecule biosynthesis	1 6↓	70↓			45↑	
response to external stimulus	28↑			31↓		1 4↓
response to pest/pathogen/ parasite	1 2↑			9↓	1 3↓	
lipid metabolism		45↑	29↓		37↑	
response to biotic stimulus	26↑			23↓		
defense response	24↑			22↓		
membrane lipid metabolism			8↓		9↑	
lipid biosynthesis		25↑			1 7↑ 9↓	
hormone metabolism		8↓			5↓	
steroid metabolism		1 6↑ 1 3↓			8↓	
C21 -steroid hormone metabolism		6↓			4↓	
cell adhesion		37↑		11↓		
alcohol metabolism		26↑			1 9↑	
muscle contraction			1 0↓	4↑		
Molecular function						
hydrolase activity	23↑	94↑	28↓	5↓	23↑	
nucleic acid binding	31↓	1 31↑	66↑	1 9↑		
RNA binding	1 0↓	36↑ ; 32↓	25↑	1 0↑		
heat shock protein activity	3↓			5↓	4↓	7↓
binding	68↓	322↑ ; 367↓		35↑		
intramolecular isomerase activity	3↑	6↓			4↓	
oxidoreductase activity	1 2↑	4↑			50↑ ; 20↓	
structural molecule activity		42↓	38↑	1 0↓		
transfera↓se activity		83↓	54↓		55↑	
complement activity		6↑	5↑			3↓
extracellular matrix structural constituent	4↓			6↓		
endopeptidase inhibitor activity		1 3↑			9↓	
enzyme inhibitor activity		20↑			1 0↓	
protease inhibitor activity		1 3↑			9↓	
protein transporter activity		1 8↑			1 3↑	
ligand-dependent nuclear receptor activity			11↓		7↑	
phosphoric ester hydrolase activity			1 9↓		1 5↑	
phosphoric monoester hydrolase activity			1 6↓		1 3↑	
protein serine/threonine phosphatase activity			5↓		5↑	
steroid hormone receptor activity			11↓		7↑	

[a] Significant biological process and molecular function categories are those with an EASE score less than 0.05.

[b] Selected differentially expressed genes were those with an FDR adjusted p-value less than 0.01 in the young v old comparison, less than 0.05 in the young AL v young CR comparison.

[c] Up regulation

[d] Down regulation

Table 10

Validation of selected microarray results by real-time QRT-PCR.

Gene name	Gene Bank accession no.		Ratio (95% confidence interval)[a]	
			Young v Old	AL v CR
RNA binding motif protein 3	AB016424	Heart: Array[b]	1.44 (1.28, 1.63)	2.37 (1.87, 2.87)
		PCR[c]	1.38 (1.11, 1.70)†	2.15 (1.65, 2.82)†
		Liver: Array	2.17 (1.34, 3.00)	2.85 (1.97, 3.73)
		PCR	2.10 (1.48, 2.97)†	2.47 (1.98, 3.07)†
		Hypo: Array		2.04 (1.69, 2.39)
		PCR		1.97 (1.53, 2.54)†
regulator of G-protein signaling 16	U94828	Heart: Array		−1.04 (−1.15, −1.08)
		PCR		1.36 (−1.12, 2.06)†
		Liver: Array		4.83 (3.21, 6.45)
		PCR		5.11 (3.55, 7.36)†
		Hypo: Array	−1.95 (−2.18, −1.72)	−1.51 (−1.70, −1.32)
		PCR	−1.47 (−1.90, −1.14)†	−1.69 (−2.19, −1.31)†
serine (or cysteine) proteinase inhibitor, clade H, member 1	X60676	Heart: Array		−1.81 (−2.30, −1.32)
		PCR		−2.44 (−3.33, −1.79)†
		Liver: Array		−1.57 (−2.07, −1.07)
		PCR		−1.79 (−2.44, −1.32)†
		Hypo: Array	−1.47 (−1.61, −1.33)	−1.60 (−1.78, −1.42)
		PCR	−1.54 (−1.92, −1.24)†	−1.60 (−2.16, −1.19)†
carnitine palmitoyl-transferase 1, liver	AF017175	Heart: Array	−1.12 (−1.22, −1.02)	1.27 (1.13, 1.41)
		PCR	−1.16 (−1.32, −1.02)†	1.65 (1.39, 1.95)†
heat shock protein, 105 kDa	L40406	Heart: Array	−1.79 (−2.02, −1.57,)	−2.11 (−2.49, −1.73)
		PCR	−2.00 (−2.34, −1.72)†	−2.88 (−3.41, −2.43)†
serine (or cysteine) proteinase inhibitor, clade A, member 3K	D00725	Liver: Array		−2.55 (−3.61, −1.49)
		PCR		−2.91 (−4.02, −2.11)†
ATP-binding cassette, sub-family D (ALD), member 2	Z48670	Liver: Array		3.17 (2.26, 4.08)
		PCR		2.94 (2.27, 3.82)†
complement component 1, q subcomponent, beta polypeptide	M22531	Hypo: Array	1.28 (1.16, 1.40)	−1.34 (−1.49, −1.19)
		PCR	1.47 (1.17, 1.85)†	−1.64 (−2.06, −1.31)†
crystallin, alpha B	AV013428	Hypo: Array	−1.58 (−1.82, −1.34)	−1.45 (−1.65, −1.25)
		PCR	−1.64 (−2.07, −1.30)†	−1.75 (−2.23, −1.37)†
Jun oncogene	X12761	Hypo: Array	−1.20 (−1.32, −1.08)	−1.33 (−1.45, −1.21)
		PCR	−1.08 (−1.35, −0.87)†	−1.33 (−1.64, −1.08)†

[a] Positive numbers indicate genes up regulated by age or CR (ratios of Old/Young or CR/AL) and negative numbers indicate genes down regulated by age or CR (ratios of Young/ Old or AL/ CR).

[b] Microarray ratios were generated by dChip and the 95% confidence intervals were generated in Excel [Young (also Young AL) n = 6; Old n =6 (Old Heart n=5); Young CR n = 6].

[c] QRT-PCR ratios are given as point estimates and the 95% confidence intervals are shown in parentheses [Young (also Young AL) n = 6; Old n =6 (Old Heart n=5); Young CR n = 6].

† QRT-PCR 95% confidence intervals overlap with their corresponding array 95% confidence intervals. In these instances, the array ratios are considered validated.