CALORIE RESTRICTION MODULATES REDOX-SENSITIVE AP-1 DURING THE AGING PROCESS

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ABSTRACT

Oxidative stress is claimed to be a major cause of aging. Recent data suggest that calorie restriction (CR) prolongs life span by its ability to retard aging, possibly by regulating the intracellular redox status through its anti-oxidative actions. Currently, there is little information showing the influences of age and CR on the redox-sensitive transcription factor activator protein-1 (AP-1). In the present study, we investigated how age affects the status of AP-1 and whether CR modulates the age effect. For our study, we used the kidney from male Fischer 344 rats, ages 6, 12, 18, and 24 months fed *ad libitum* (AL) or a CR diet. Results from our study showed that AP-1 binding activity markedly increases with age, while CR keeps this activity at the level of 6-month-old rats. We found that c-Jun and c-Fos protein levels increase during aging, and that aging induces phosphorylation of c-Jun, which might enhance AP-1 transcriptional activity. For CR's action, we found that in the nucleus of aged rats, AP-1 activation was blunted by decreasing c-Jun and c-Fos levels and inhibiting c-Jun protein phosphorylation. Results also indicated that matrix metalloproteinase-13 and heme oxygenase-1, which have an AP-1 binding site in their promoter regions, have a similar tendency toward AP-1 binding activity. Based on the data of these findings, we concluded that AP-1 activity increases in rat kidney with age and that CR reduces AP-1 activity.

INTRODUCTION

Much evidence strongly indicates that most age-related changes are causally linked to deleterious reactive oxygen species (ROS), the incessant, damaging products generated from aerobic metabolism (1-3). Recent work using molecular probes revealed that the majority of intracellular signaling systems are exquisitely sensitive to changes in the redox condition(4). The currently

most popular theory of aging, the oxidative stress hypothesis of aging, proposes that an imbalance in the redox status due to excessive oxidative forces leads to cellular dysfunctions and pathogenesis during aging (3,4). With time, oxidatively altered DNA, proteins, lipids, and other major cellular components, including cell membranes and anti-oxidative defense systems, cause a disruption of homeostasis resulting in many age-related degenerative diseases (3,4). One such phenomenon showing an age-related deleterious process strongly implicates an oxidatively induced pro-inflammatory process, which was recently proposed by our laboratory (See review, ref. 5)

Recent work on calorie restriction (CR) provides abundant evidence that CR exerts diverse benefits by its ability to stave off age-related oxidative stress through its remarkable anti-oxidative properties (3,6-9). CR has been shown to provide a variety of protective mechanisms against oxidative damage and, at the same time, uphold antioxidant defense systems, including major ROS scavengers during aging (3). Based on these and other data, it has been proposed that these mechanisms, which effectively suppress ROS, are responsible for CR's powerful anti-aging action (3,9).

Recent molecular explorations of the aging process have focused on the oxidative modulation of cellular signaling systems and on alterations in gene regulation and expression (10). Most gene regulation and expression activities are well known to be controlled by oxidatively sensitive transcription factors that undergo age-related changes (11,12).

Among many transcription factors, the activator protein-1 (AP-1) transcription factor is exquisitely sensitive to the intracellular redox state (13) because of the thiol-moiety of its peptides. AP-1 is a complex of oncogene proteins of the Jun and Fos families. Three mammalian Jun proteins (c-Jun, JunB, and JunD) and four Fos family members (c-Fos, Fra-1, Fra-2, and FosB) have been identified (14). AP-1 proteins, the Jun-Jun homodimers or the Jun-Fos heterodimer, bind to TPA-response elements (TRE) or AP-1 binding sites (5'-TGAG/CTCA-3') present in the promoter region of genes implicated in cell proliferation, differentiation, inflammation, and stress response (15).

The regulation of AP-1 activity occurs at two levels, either transcriptionally or post-translationally (16). The

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quantity of AP-1 proteins is usually regulated by controlling the transcription of *jun/fos* genes (16). The induction of *c-fos* transcription occurs rapidly, i.e., transiently, from stimuli such as cytokines, cAMP, growth factors, and UV (17). The activities of both pre-existing and newly synthesized AP-1 are modulated through their phosphorylation. The phosphorylation of c-Jun at Ser-73 and Ser-63, located within its transactivation domain, potentiates its transcriptional activity (18). Like c-Jun, c-Fos is phosphorylated at Thr-232, thereby potentiating its transcriptional activity (19). Mitogen-activated protein kinases (MAPKs) signaling pathways are known to influence AP-1 activity by both increasing the abundance of AP-1 components and stimulating their activity directly (12).

Although age-dependent changes in AP-1 binding activity have been reported previously, a controversy exists over the relation between aging and AP-1 activity because of the tissues and cells tested (20,21). To date, no reports give molecular insights into AP-1 regulation by aging and CR in the kidney.

In the present study, we delineate the molecular events involved in the DNA binding activity of the AP-1 transcription factor and define its components as a function of age. Furthermore, we expanded our investigation on the mechanism underlying the regulation of AP-1 age-related changes by utilizing CR's anti-oxidative ability.

RESULTS

Changes in AP-1 Binding Activity by Aging and CR

To determine the extent of age-related activation of AP-1, EMSA was carried out using nuclear proteins. Figure 1A shows age-induced increases in the nuclear binding activity of AP-1 in the *ad libitum* (AL) group, while little change was detected in CR rats, even in senescent, 24-month-old rat kidney. Binding specificity of the AP-1 complex was demonstrated using a 100-fold excess of an unlabeled oligonucleotide, which competed for binding (Figure 1A, Lane 10). These data clearly showed that aging enhances AP-1 activation and that CR attenuates the age-related increase.

Supershift Assay with Antibodies against AP-1 Subunits

To identify the components of the AP-1 complex, supershift assays were performed using antibodies against AP-1 subunits (Figure 1B). The data showed a shifting of the AP-1-DNA complex by the addition of antiserum against c-Jun or c-Fos subunit (lane 3, 4). These findings identified both c-Jun and c-Fos as part of age-modulated AP-1 binding activity.

Effect of Age and CR on Nuclear c-Jun and c-Fos Protein Levels

To verify the possibility that increased c-Jun and c-Fos proteins in nucleus caused increased AP-1 DNA-binding during aging, we examined nuclear protein levels by Western blot analysis using c-Jun- or c-Fos-specific antibodies. Results in Figure 2 show that nuclear levels

Figure 1: CR suppresses age-related increases of AP-1 binding activity. (A) The EMSA method was used to compare nuclear AP-1 binding activities in kidney nuclear protein from AL or CR rats, ages 6, 12, 18, and 24 months. Lane 1, probe without nuclear protein sample; Lane 2-9, kidney nuclear protein samples of AL or CR rats, ages 6, 12, 18, and 24 months, respectively. Lane 10, competition assay using a 100-fold excess of an unlabeled AP-1 oligonucleotide. One representative result is shown from three experiments that yielded similar results. The level of AP-1 DNA binding was quantified by densitometry. The data shown are presented as a percent of a 6-month-old AL rat. Results of two-factor ANOVA: age main effect, $p < 0.001$; diet main effect, $p < 0.001$, and age and diet interaction, $p < 0.001$. Results of one-factor ANOVA: $^{*}p < 0.01$, $^{***}p < 0.001$ vs. 6-month-old AL rat; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. age-matched AL rats. (B) Supershift experiment using antiserum against the c-Jun and c-Fos were performed. Lane 1, probe without nuclear protein; Lane 2, nuclear protein sample of aged rat; Lane 3 and 4, nuclear protein sample of aged rat incubated with anti-c-Jun and c-Fos, respectively.

of c-Jun and c-Fos increased with age in AL rats and that CR prevented these changes (Figure 2A and B). Thus, we confirmed that age-related increases of nuclear c-Jun and c-Fos contributed to the increased AP-1 binding activity in AL rats, while CR prevented AP-1 binding activity by reducing the nuclear levels.

Effect of Age and CR on c-Jun and c-Fos mRNA Levels

To determine whether there might be alterations in the gene expression of *c-jun* and *c-fos* mRNAs during aging, the RT-PCR assay was performed to measure the abundance of *c-jun* and *c-fos* mRNAs in young and old AL and CR rats. As shown in Figure 3, aging induced higher *c-jun* and *c-fos* mRNA levels in AL rats, while CR rats retained lower levels than AL rats. Protein levels confirmed these results as shown in Figure 2.

Figure 2: Effect of age and CR on nuclear protein levels of c-Jun and c-Fos. Western blot analysis was performed to investigate renal nuclear c-Jun (A) and c-Fos (B) protein levels in nuclear protein extracts (25 µg protein) from AL or CR rats, ages 6, 12, 18, and 24 months. One representative blot of each protein is shown from three experiments that yielded similar results, respectively. The levels of each protein were quantified by densitometry. The data shown are presented as a percent of a 6-month-old AL rat. Results of two-factor ANOVA: (A) nuclear c-Jun protein level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$), and age and diet effect ($p < 0.001$); (B) nuclear c-Fos protein level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$) and age and diet effect ($p < 0.001$); (C) nuclear HIF-1β protein level is loading control for Western blot. Results of one-factor ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 6-month-old AL rat; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. age-matched AL rats.

Figure 3: Effect of age and CR on mRNA levels of *c-jun* and *c-fos*. RT-PCR analysis of renal *c-jun* (A) and *c-fos* (B) were performed from RNA isolated from AL or CR rats, ages 6, 12, 18, and 24 months. GAPDH primers were used in separate PCR reactions to control for the efficiency of cDNA synthesis in each sample. One representative result is shown from three experiments that yielded similar results. The level of α -tubulin mRNA was quantified by densitometry. The data shown are presented as a percent of a 6-month-old AL rat. Results of two-factor ANOVA: (A) *c-jun* mRNA level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$), and age and diet effect ($p < 0.05$); (B) *c-fos* mRNA level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$) and age and diet effect ($p < 0.001$). Results of one-factor ANOVA: * $p < 0.05$, ** $p < 0.01$ vs. 6-month-old AL rat; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. age-matched AL rats.

Effect of Age and CR on Phosphorylation of Nuclear c-Jun

Phosphorylation of c-Jun at Ser-73 and Ser-63, located within its transactivation domain, potentiates transcriptional activity (18). To examine the possibility that in-

creased phosphorylation of c-Jun in nucleus caused increased AP-1 DNA-binding during aging, we measured phospho-c-Jun levels by Western blot analysis using phospho-c-Jun-specific antibody. As shown in Figure 4, phospho-c-Jun protein increased with aging in AL rats, and CR rats showed lower amounts than AL rats, indicating that aging induces phosphorylation of c-Jun, which caused the increases of AP-1 DNA-binding activity.

Figure 4: CR suppresses age-related phosphorylation of c-Jun. Kidney nuclear extracts were subjected to Western blot with an antibody against phospho-c-Jun. One representative result is shown from three experiments that yielded similar results. The level of phospho-c-Jun was quantified by densitometry. The data shown are presented as a percent of 6-month-old AL rat. (A) Two-factor ANOVA indicates that phospho-c-Jun is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$), and age and diet interaction ($p < 0.001$); (B) nuclear HIF-1 β protein level is loading control for Western blot. Results of one-factor ANOVA: ** $p < 0.01$, *** $p < 0.001$ vs. 6-month-old AL rat; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. age-matched AL rats.

Effect of Age and CR on AP-1-Dependent Gene Expression of MMP-13 and HO-1

To ascertain whether the changes we detected in AP-1 DNA binding activity during aging are in fact correlated with AP-1-dependent gene expression, we examined gene expressions in the matrix metalloproteinase-13 (MMP-13) and heme oxygenase-1 (HO-1). These two genes, which contain an AP-1 binding site in their promoter regions, are known to be controlled by AP-1

Figure 5: Effect of age and CR on gene expression of MMP-13 and HO-1. Western blot analysis was performed to investigate renal nuclear MMP-13 (A) and HO-1 (B) protein levels in postmitochondrial protein fractions (100 μ g protein) from AL or CR rats, ages 6, 12, 18, and 24 months. RT-PCR analysis of renal *MMP-13* (C) and *HO-1* (D) were performed from RNA isolated from the kidney of AL or CR rats. GAPDH primers were used in separate PCR reactions to control for the efficiency of cDNA synthesis in each sample. One representative result is shown from three experiments that yielded similar results. The data were quantified by densitometry, and presented as a percent of a 6-month-old AL rat. Results of two-factor ANOVA: (A) MMP-13 protein level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$), and age and diet effect ($p < 0.001$); (B) HO-1 protein level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$) and age and diet effect ($p < 0.001$); (C) MMP-13 mRNA level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$) and age and diet effect ($p < 0.001$); (D) HO-1 mRNA level is significantly changed by the age effect ($p < 0.001$), diet effect ($p < 0.001$) and age and diet effect ($p < 0.001$). Results of one-factor ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. 6-month-old AL rat; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. age-matched AL rats.

regulation (22,23). Our test shows that mRNA and protein levels of MMP-3 and HO-1 are well-correlated with AP-1 activity (5). From these results, age-related changes in AP-1 activity are deemed to contribute to MMP-13 and HO-1 gene expression alterations.

DISCUSSION

Much of the recent evidence strongly implicates oxidative stress as a major factor contributing to the aging process (1-3). Moreover, the well-known anti-aging action of calorie restriction (CR) has been proposed to be the main mechanism underlying the retardation of aging processes (3, 6-9).

The current study was designed to explore the molecular mechanisms of alterations to the redox-sensitive nuclear AP-1 transcription factor during aging and its modulation by CR. We were able to document that the aging process causes the up-regulation of AP-1 activity and that CR counteracted this up-regulation. Based on what is known about increased oxidative stress in aged animals (3,7), it is reasonable to expect that the age-related up-regulation of redox-sensitive AP-1 is likely caused by a shift in intracellular redox balance.

The modification of AP-1 activity can take place at two levels: transcriptional and post-translational regulation (16). First, the amount of AP-1 proteins is regulated by controlling gene transcription (16). The *c-jun* and *c-fos* genes, which are expressed usually at low levels, can turn on their expression and rapidly elevate in response to many stimuli (17). Second, the level of regulation by phosphorylation of c-Jun at Ser-73 and Ser-63, and c-Fos at The-232 domain potentiates AP-1 transactivation transcription activities (18).

Our present data, shown in Figure 2, confirmed that age-related increases of nuclear c-Jun and c-Fos contribute to increased AP-1 binding activity. Furthermore, aging was also shown to induce the phosphorylation of c-Jun (Figure 4), which led to further increase of the binding activity. Our data on CR showed the prevention of AP-1 activation by a decrease in the nuclear levels of c-Jun and c-Fos and by a reduction of the phosphorylation of the c-Jun protein in the nucleus of aged rat kidney.

Existing evidence shows that mitogen-activated protein kinases (MAPKs) signaling pathways influence AP-1 activity by both increasing the amount of AP-1 components and stimulating their activity directly (16). Three subfamily components of MAPKs, extracellular signal-related kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 MAPK, are each known to contribute to the AP-1 induction in response to a diverse array of extracellular stimuli (16). Our laboratory recently reported (12) that the aging process strongly enhanced all three MAPKs activities in rat kidney and that CR markedly suppressed the age-related activation of MAPKs in this tissue. Based on our own and other findings, it is highly probable that the age-related increases of MAPKs activity enhanced the AP-1 binding activity during aging in the AL rats.

It is worth noting some contradicting data in the literature on the changes in AP-1 activity during aging. An age-dependent increase in AP-1 binding activity has been reported in rat liver (24) and gastric mucosa (25), while other authors have described a decrease in AP-1

binding activity in rat hypothalamus (26), hippocampus (27), and frontal cortex (27). Further, decreased AP-1 activity was reported in human diploid fibroblast (28), T lymphocytes (29), and vascular endothelial cells (30), but increased activity was noted in vascular smooth muscle cells (31). Taking the available data together, at present, including our present data, the effect of age on the AP-1 binding activity appears to be tissue- and cell-specific, and further investigations are warranted.

The modulation of the AP-1 status is well known to be influenced by various stimuli, including growth factors, cytokines, tumor promoters, carcinogens, and the increased expression of various oncogenes, such as *src* and *ras* (32). Enhanced levels in the expression of AP-1-dependent genes were found in tumors derived from both in vivo and in vitro transformation studies (32). Also, the role of AP-1 in tumor cell invasion is documented by increased AP-1-dependent gene expression, such as matrix metalloproteinases (33). Moreover, wide implications for AP-1 became known in the pathogenesis for proteinuric renal disease (34), Alzheimer disease (35), hepatitis B infections (36) and HIV (37). A recent review published from our laboratory on the interrelation of inflammation and aging highlights the important role of redox-sensitive transcription factors in normal and pathologic conditions (5).

In conclusion, our present study is the first report showing evidence of the up-regulation of AP-1, including its binding activity by age and its suppression by CR in rat kidney. Our data suggest that changes in AP-1 may well be a response to cellular stress and that the uncontrolled activation of AP-1, as seen in old AL animals, is likely to lead to functional deteriorations with the passage of time.

EXPERIMENTAL PROCEDURES

Animals

Rat maintenance procedures for specific-pathogen free status and the dietary compositions for chow and CR regimens have been previously reported (6). This study complied with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (Publication No.85-23) and was approved by an Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Briefly, male, specific pathogen-free Fischer 344 rats were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% α -methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-Floc. The *ad libitum* (AL) fed group had free access to both food and water. The animals designated as CR were fed 60% of the food intake of their AL-fed littermates, beginning at 6 weeks of age. Rats at 6, 12, 18, and 24 months of age were sacrificed by decapitation and the kidneys were quickly removed and rinsed in iced-cold buffer (100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethyl-sulfonylfluoride (PMSF), 1 μ M pepstatin, 2 μ M leupeptin, 80 mg/L trypsin inhibitor, 20 mM β -glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate (pH 7.4). Tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Close attention was given to histopathological examination of the aged kidney, and no evidence of nephrotic lesions was found in these soy protein-fed Fischer rats even at the advanced age of 24 months as shown by Iwasaki et al. (38).

Materials

All chemical reagents were obtained from Sigma, except where noted. Western blotting detection reagents were obtained from Amersham (Bucks, UK). RNAzol™ B was obtained from TEL-TEST, Inc. (TX, USA). Reverse transcriptase was from Gibco-Bethesda Research Laboratory (MD, USA), Taq DNA polymerase and 10X PCR buffer were from Perkin Elmer (CA, USA). Antibody against c-Jun was from Transduction Laboratories (KY, USA). Antibodies against c-Fos and phospho-c-Jun were obtained from Santa Cruz Biotechnology (CA, USA). Anti-rabbit IgG-horseradish peroxidase-conjugated antibody and anti-mouse IgG-horseradish peroxidase-conjugated antibody were obtained from Amersham (Bucks, UK). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (MA, USA). All other materials were obtained in the highest available grade.

Primers

Primers for reverse transcriptase-polymerase chain reaction (RT-PCR) were synthesized by Bioneer (Daejeon, Korea) and had the following sequences (the expected size are given in parentheses).

Rat c-jun: sense, 5'-AGTTCTTGTCCTCAAGAAC-3'; anti-sense, 5'-ATGCGGTTTCTCATGCGCTT-3' (514 bp)

Rat c-fos: sense, 5'-CACAGGACTTTTGCGCAGAT-3'; anti-sense, 5'-CTCAGGGTCATTGAGAAGAG-3' (608 bp)

Rat MMP-13 (39): sense, 5'-AAAGAACATGGTGACTTCTACC-3'; anti-sense 5'-ACTGGATTCTTGAACGTC-3' (243 bp)

Rat HO-1: sense, 5'-CTTGATACCATATCTATACGGC-3'; anti-sense, as 5'-TAGTATCTTGAACCAGGCTAG-3' (584 bp)

Rat GAPDH: sense, 5'-GGGTGATGCTGGTGCTGAGTATGT-3'; anti-sense 5'-AAGAATGGGAGTTGCTGTTGAAGTC-3' (617 bp)

Tissue Preparation

One gram of kidney was homogenized with 5 ml of homogenate buffer containing 100 mM Tris, containing 20 mM β -glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate, 1 mM EDTA, 0.5 mM PMSF, 1 μ M pepstatin, 80 mg/L trypsin inhibitor, pH 7.4, and centrifuged at 900 g at 4°C for 15 min. The supernatants were re-centrifuged at 12,000 g at 4°C for 15 min to yield a sedimented mitochondrial fraction and a postmitochondrial supernatant fraction. The supernatants were centrifuged again at 105,000 g at 4°C for 1 hr to separate cytosol supernatant fraction.

Preparation of Nuclear Extracts

All solutions, tubes, and centrifuges were maintained at 0-4°C. For each nuclear extract preparation, three rat kidney tissues were pooled. The preparation of nuclear

extracts was based on previous methods (40). The nuclear extract was frozen at -80°C in aliquots until the electrophoretic mobility shift assay (EMSA, see below) was done. The total protein concentration in samples was measured with a Sigma protein assay reagent kit containing bicinchoninic acid.

Electrophoretic Mobility Shift Assay

The EMSA method was used to characterize the binding activities of AP-1 transcription factors in nuclear extracts (41). AP-1 oligonucleotide was 5'-CTAGTGA-TGAGTCAGCCGGATC-3'. Protein-DNA binding assays were performed with 10 μ g of nuclear protein. To minimize salt on binding, the concentration of salt was adjusted to the same level in all samples. Unspecific binding was blocked by using 1 μ g of poly(dI-dC)•poly(dI-dC). The binding medium contained 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 1.0% Nonidet P40 (NP40), and 10 mM Tris/HCl, pH 7.5. In each reaction, 20,000 cpm of a radiolabelled probe was included. Samples were incubated at room temperature for 20 min, and the nuclear protein-³²P-labelled oligonucleotide complex was separated from free ³²P-labelled oligonucleotide by electrophoresis through a 5% native polyacrylamide gel in a running buffer containing 50mM Tris, pH 8.0, 45mM borate and 0.5mM EDTA. After separation was achieved, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film for 1-2 days at -80°C.

To determine the specificity of the nuclear protein binding, competitions were carried out under the same conditions using a 100-fold molar excess of the unlabeled NF- κ B oligonucleotide probe. For supershift analysis, 5 μ g of nuclear extracts were incubated on ice for 30 min with an antibody against AP-1 subunits at a 5.0 μ g/ μ l final concentration, prior to addition of the radiolabelled probe.

Western Blotting

Western blotting was carried out as described previously (42). Homogenized samples were boiled for 5 min with a gel-loading buffer (0.125 M Tris-Cl, 4% SDS, 10% 2-mercaptoethanol, pH 6.8, 0.2% bromophenol blue) in the ratio of 1:1. Total protein-equivalents (100 μ g) for each sample were separated by SDS-PAGE using 10% acrylamide gels as described by Laemmli (43), and transferred to PVDF membrane at 15 V for 1 hr in a semi-dry transfer system. The membrane was immediately placed into blocking buffer (1% non-fat milk) in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. The blot was allowed to block at room temperature for 1 hr. The membrane was incubated with anti-c-Jun, c-Fos, phospho-c-Jun, MMP-13, or HO-1 for 1 hr at 25°C, followed by an anti-rabbit or -mouse IgG-horseradish peroxidase-conjugated antibody for 1 hr at 25°C. Antibody labeling was detected using enhanced chemiluminescence per the manufacturer's instructions. Pre-stained protein markers were used for molecular weight determinations.

Isolation of RNA

Total RNA was isolated by the method of Puissant and Houdebine (39). Briefly, tissue samples were homog-

enized in the presence of RNAzol™ B (2 ml per 100 mg tissue) with several strokes in a tissue homogenizer. Aliquots of 0.2 ml chloroform per 2 ml homogenate were added. The samples were shaken vigorously for 15 sec, and kept in ice for 5 min. The suspension was centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, to which an equal volume of isopropanol was added, and the samples were kept at 4°C for 15 min. Samples were again centrifuged at 12,000 g for 15 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequently centrifuged at 7,500 g for 8 min at 4°C. The pellet was dried for 10-15 min. The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water.

Assay of RT-PCR

(a) Reverse Transcription: The first strand cDNA was synthesized from 2 µg of total RNA. DEPC-treated water and 250 ng random primer were added, incubated for 5 min at 75°C, and incubated on ice for 5 min. Aliquots of 2 µl 0.1 M DTT, 4 µl 5X buffer, 4 µl 2.5 mM dNTP, 100 U reverse transcriptase, and 16.5 U of RNase inhibitor were added and incubated for 2 hr at 37°C. The reaction was stopped by boiling for 2 min at 100°C, and cDNA was stored at -20°C until use.

(b) PCR: To carry out the PCR, 25 µl PCR master mix was added to each tube directly. Deoxynucleotides were added to a final concentration of 0.2 mM. A PCR buffer of 10X was diluted in ratio of 1:10. One point two five units of Taq DNA polymerase was added. The reaction medium was then added, 50 µg of sense and antisense primers for *c-jun*, *c-fos*, MMP-13, HO-1, or GAPDH to each tube, respectively. Reaction conditions consisted of 31 cycles for rat *c-jun*, 29 cycles for rat *c-fos*, 35 cycles for rat MMP-13, 31 cycles for rat HO-1, and 20 cycles for GAPDH at 94°C for 30 sec denaturation, at 54°C for 30 sec annealing, and at 72°C for 1 min extension. Electrophoresis was performed in 1% agarose gel. After staining in ethidium bromide solution was achieved, the gel was observed under UV transilluminator.

Statistical Analysis

The statistical significance of the differences between the treatments (age and diet) was determined by two-factor ANOVA. One-factor ANOVA was conducted to analyze significant differences among all possible age and diet pairs. Differences among the mean value of individual groups were assessed by the Fischer's Protected LSD post-hoc test. Values of $p < 0.05$ were considered statistically significant.

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ABBREVIATIONS

AP-1, activator protein-1; ROS, reactive oxygen species; CR, calorie restriction; RT-PCR, reverse tran-

scriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay.

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