Modulation of PPAR in Aging, Inflammation, and Calorie Restriction

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Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor superfamily of transcription factors, are key regulators in various pathophysiological processes related to energy metabolism including lipid, carbohydrate metabolism, and inflammation. At present, little information is on the effect of age and calorie restriction (CR) on PPARs. In the present study, we investigated how age and CR (60% of the ad libitum intake) modulate PPARs in kidneys obtained from Fischer 344 rats, ages 13 and 25 months. Results showed that nuclear protein, mRNA level, and DNA binding activity of PPARs decreased with age, while CR blunted the reduction. Our findings were verified in separate experiments in which rats were injected with lipopolysaccharide, with the result of increased susceptibility to inflammation. Based on these findings, we conclude that the altered expression of PPARs may be due to increased oxidative stress with age, and that CR prevents these decreases through its antioxidative action.
regulated proinflammatory molecules such as tumor necrosis factor (TNF-α and TNFβ), interleukins (IL-1β, IL-2, and IL-6), chemokines (IL-8 and RANTES), adhesion molecules (ICAM-1, VCAM-1, and E-selectin), and that enzymes such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) increased with age (13).

Calorie restriction (CR), a potent experimental paradigm for the retardation of aging, has been shown to exhibit broad and effective antioxidative properties (12). Recent research provides strong evidence that CR may exert diverse anti-aging benefits by its ability to modulate age-related oxidative stress and by its antiinflammatory effect (14,15). Several studies reported that CR attenuates age-related increases of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in myocardial ischemia-reperfusion injury and other diseases (16–18). Based on the data from these studies and others, the beneficial effects of CR can be viewed as an outcome of the proper maintenance of cellular signal transduction activities through the modulation of various redox-sensitive transcription factors. Supporting this notion, our laboratory recently reported that the binding activity of redox-sensitive transcription factors, AP-1, NF-κB, and HIF-1, which DNA-increased during the aging process, was effectively suppressed by CR (10).

Much is known about the activation of PPARs via PPREs and various PPRE participations, but far less information exists for the mode of PPAR modulation during aging. Although, the aging research literature does show that the activity and metabolism of peroxisomes decline (19,20) and that expression of PPARα is reduced during the aging process (21,22), to date, no data are reported on the molecular regulation of PPARγ and PPARβ by CR during the aging process.

In the present study, we attempted to document age-related changes in the expression and activity of PPARα and PPARγ and the extent of their modulation by CR in aged rat kidney. In addition, we present data on lipopolysaccharide (LPS)-induced inflammatory reaction to further characterize the modulation of PPARα and PPARγ under acute inflammatory conditions.

**Experimental Procedures**

**Animals and LPS Treatment Procedure**

**Animals.**—Rat maintenance procedures for specific pathogen-free (SPF) status and the dietary compositions for chow and CR regimens have been previously reported (23). This study complies with the Guide for the Care and Use of Laboratory Animals published by the 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 SPF Fischer 344 rats were fed a diet of the following composition: 21% soybean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% l-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 of their AL-fed littermates, beginning at 6 weeks of age. Rats at 16 and 25 months of age were killed by decapitation, and the kidneys were quickly removed and rinsed in ice-cold buffer (100 mM Tris, 1 mM EDTA, 0.2 mM phenylmethyl-sulfonyfluoride [PMSF], 1 μM pepstatin, 2 μM 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.

**LPS treatment.**—SPF male Fischer 344 rats at 13 and 31 months of age were separated into young (n = 6) and old (n = 6) groups, respectively. To investigate the effects of inflammation on the aging process, two additional groups were used for the injection of bacterial LPS. LPS was injected intraperitoneally with 5 mg/kg body weight doses in young (n = 6) and old (n = 6) rats. After 5 hours, rats were killed by decapitation and the kidneys quickly removed. The tissue was immediately immersed in liquid nitrogen and stored at −80°C (24).

**Primers.**—Primers for reverse transcriptase-polymerase chain reaction (RT-PCR) were synthesized by Bioneer (Daejeon, Korea) and had the following sequences (the expected sizes are given in parentheses). rat PPARγ: sense, 5′-GGTGATCTTAACTGCGG-3′; antisense, 5′-CAT-GGACACCATACCTGAGC-3′ (530 bp), rat PPARα: sense, 5′-GATGACCTGGAAAGTCCTTT-3′; antisense, 5′-AGGTAGCTCGTGAGCT-3′ (595 bp) and rat GAPDH: sense, 5′-GGGTATGCCTGGCTGCTGATT-3′; antisense 5′-AAGAATTGGGATTGGTGGAA-GTC-3′ (617 bp).

**Preparation of nuclear extracts.**—All solutions, tubes, and centrifuges were maintained at 0°C–4°C. For each nuclear extract preparation, three rat kidney tissues were pooled. The preparation of nuclear extracts was based on previous methods (25). All buffers contained 2 μg/ml of each protease inhibitor: aprotonin and leupeptin and 0.5 mM PMSF. Two grams of rat kidney harvested from young and old rats, were minced in phosphate-buffered saline. Minced tissue was incubated for two 15-minute intervals in relaxation buffer 1 (RBI; 100 mM KCl, 5 mM MgCl2, 5 mM EGTA, 5 mM sodium pyrophosphate, pH 6.8), followed by two 10-minute washes in RBII (50 mM KCl, 5 mM MgCl2, 1 mM EGTA, 1 mM sodium pyrophosphate, pH 6.8). The tissue was then washed with homogenization buffer A: 0.3 M sucrose, 0.15 M KCl, 0.15 M spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.5, 0.5 mM PMSF, 2 mM dithiothreitol (DTT), followed by homogenization in fresh homogenization buffer A. Kidney tissue was disrupted with homogenizer. Kidney homogenates were then centrifuged at 1000 g for 10 minutes in a Beckman JA-25-50 rotor (Beckman, Fullerton, CA), and the pellets were solubilized in homogenization buffer B (0.3 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM HEPES, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 1 mM DTT) containing 0.5% (v/v) Triton X-100. The solubilized pellet was homogenized, followed...
by homogenization with a hand-held Teflon glass Dounce homogenizer. The nuclear pellet was solubilized in 10 ml of lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 10% [w/v] glycerol). Nuclei were lysed by drop-wise addition of 3 M NH₄SO₄ (pH 7.9) to a final concentration of 0.4 M, and the resulting extract was gently shaken for 30 minutes at 4°C. The lysate was ultracentrifuged at 126,000 g for 1 hour, and the supernatant was collected. Solid NH₄SO₄ (0.3 g/ml) was added slowly to the supernatant, and nuclear proteins were allowed to precipitate on ice for 30 minutes. Precipitated proteins were pelleted by ultracentrifugation at 126,000 g for 30 minutes and resuspended in 500 μl of dialysis buffer (25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% [w/v] glycerol, 1 mM DTT). The muscle nuclear protein extract was dialyzed twice for 1 hour each time against dialysis buffer, and aliquots were quick frozen and stored at –80°C in aliquots until the electrophoretic mobility shift assay (EMSA, see below) was done. Once thawed, nuclear protein extracts were not refrozen for use. The total protein concentration in samples was measured with a Sigma (St. Louis, MO) protein assay reagent kit containing bicinchoninic acid.

Electrophoretic mobility shift assay.—The EMSA method was used to characterize the binding activities of PPAR in nuclear extracts (26). PPRE oligonucleotide was 5’-CATAAACTAGGTCAAAGGTCA-3’. Protein-DNA binding assays were performed with 10 μg of nuclear protein. To minimize the effect of salt on binding, the concentration of salt was adjusted to the same level in all samples. Nonspecific binding was blocked by using 1 μg of poly(dI-dC)·poly(dI-dC). The binding medium contained 5% glycerol, 1 mM MgCl₂, 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 radio-labelled probe was included. Samples were incubated at room temperature for 20 minutes, 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 30 mM Tris, pH 8.0, 45 mM borate, and 0.5 mM EDTA. After separation was achieved, the gel was vacuum-dried for autoradiography and was exposed to Fuji X-ray film (Seoul, Korea) 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 PPRE oligonucleotide probe.

Western blotting.—Western blotting was carried out as described previously (27). Homogenized samples were boiled for 5 minutes with a gel-loading buffer (0.125 M Tris-Cl, 4% SDS, 10% 2-mercaptoethanol, pH 6.8, 0.2% bromphenol blue) at a ratio of 1:1. Total protein-equivalent samples were separated by SDS-polyacrylamide gel as described by Laemmli (28), and transferred to poly(vinylidene fluoride) membrane at 15 V for 1 hour in a semidry transfer system. The membrane was immediately placed into blocking buffer (5% nonfat milk) in TBS-T buffer (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 hour. The membrane was incubated with anti-PPARγ and anti-PPARα for 1 hour at 25°C, followed by an antirabbit immunoglobulin G (IgG)-horseradish peroxidase-conjugated antibody for 1 hour at 25°C. Antibody labeling was detected using enhanced chemiluminescence per the manufacturer’s instructions. Prestained protein markers were used for molecular weight determinations.

Isolation of RNA.—Total RNA was isolated using TRIzol reagent (Gibco-BRL, Gaithersburg, MD), as per the manufacturer’s instructions. Briefly, tissue samples were homogenized in the presence of Trizol (2 ml per 100 mg tissue) with several strokes in a tissue homogenizer. Aliquots of 0.2 ml chloroform per 1 ml homogenate were added. The samples were shaken vigorously for 15 seconds, and kept in ice for 5 minutes. The suspension was centrifuged at 12,000 g for 15 minutes 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 minutes. Samples were again centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by vortexing and subsequently centrifuged at 7500 g for 8 minutes at 4°C. The pellet was dried for 10–15 minutes. The RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water.

Assay of RT-PCR.—Two micrograms of total RNA were used to generate a cDNA template for RT-PCR. The first strand synthesis was performed using random hexamers (Promega, Madison, WI) and Superscript II reverse-transcriptase (Invitrogen, Carlsbad, CA). The first-strand cDNA products were further diluted and used as the PCR template. The PCR reaction in a total volume of 25 μl, containing 0.25 units of Taq polymerase, 0.25 mM dNTPs, PCR buffer (PE Applied Biosystems, Foster City, CA), and 50 ng of the relevant oligonucleotide primers. Parallel amplifications (20, 23, 26, and 29 cycles) of a given cDNA were used to determine the optimum number of cycles. For each gene under study, a readily detectable signal within the linear range was observed after 29 cycles. For the actual analysis, samples were heated for 5 minutes at 94°C, and then 23 cycles were carried out, each consisting of 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. This was followed by a final 10-minute extension at 72°C. In this study, GAPDH was used as the expression control. PCR reaction products were separated on a 1.2% agarose gel and visualized under ultraviolet light after ethidium bromide staining.

Statistical analysis.—For Western blotting and RT-PCR, data from three independent experiments were collected. The statistical significance of the differences between the treatments (age and diet) was determined by the analysis of variance (ANOVA) method. 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
RESULTS

Effect of Age and CR on PPARα and PPARγ mRNA Levels

To determine gene expression alterations in PPARα and PPARγ during aging, the RT-PCR assay was carried out to measure the abundance of PPARs mRNAs in young (13 months) and old (25 months) AL and CR rats. As shown in Figure 1, we observed a slight decrease in PPARγ (Figure 1A) and PPARα (Figure 1B) levels in mRNA. Compared with young AL rats, old AL rats showed decreased PPARα and PPARγ mRNA levels of 30% and 46%, respectively. However, old CR rats showed higher levels of both PPARα and PPARγ than their AL counterparts.

Effect of Age and CR on Nuclear PPARα and PPARγ Protein Levels

To verify the possibility that a reduction of PPARα and PPARγ levels in mRNA causes a decline in PPARα and PPARγ protein levels during the aging process, we examined nuclear protein levels by Western blot analysis using PPARα- and PPARγ-specific antibody. The results in Figure 2 show that nuclear levels of PPARγ (Figure 2A) decreased with age in AL rats (84% decrease) and that CR (26% decrease) ameliorated this change. Likewise, aging markedly decreased nuclear levels of PPARα (Figure 2B). Compared with young AL rats, we observed a 53% decrease in old AL rats; however, the decrease (23%) was deterred in old CR rats.

Changes in PPAR Binding Activity by Aging and CR

We performed EMSA on rat kidney PPAR-DNA binding to examine whether a change in renal PPARs expression affects PPAR-DNA binding to PPRE, which is in the PPAR-binding domain. Figure 3A shows the film from the EMSA of PPAR-DNA binding in the AL and CR rats. Using a PPRE oligonucleotide, PPAR-DNA binding activity was significantly lower in old (25 months) compared with young (13 months) rats in the AL groups. We found that PPAR-DNA binding activity decreased by 64% in the old AL groups compared with the young (Figure 3B). However, old CR rats showed higher (19% decreased) activity levels.
compared with their age-matched AL group. These data clearly showed that PPAR activation declines with age and that CR attenuates this age-related decline.

**PPARα and PPARγ Expression Changes During Aging and LPS Challenge**

To duplicate our findings and to further understand the association between alterations of PPARs and the inflammatory response during aging, groups of young and old rats were challenged with a potent inflammatory stimulus, LPS. First, we used RT-PCR to measure mRNA levels in LPS-treated young and old rats. The results shown in Figure 4 indicate decreased concentrations of PPARγ (Figure 4A) and PPARα (Figure 4B) in old (31-month-old) rats compared with young (13-month-old) rats, not only in the control group but also in the LPS-treated group, particularly, the old LPS-treated rats, which showed the lowest amount of PPARs among all groups.

**Effect of Age and LPS-Induced Inflammation on Nuclear PPARγ Protein Level**

Previous studies report a decrease in PPARα with age and inflammatory stimuli in various tissues (21,22,29). In this study, we investigated alterations of PPARγ with age. To determine whether aging and LPS-induced inflammation influence PPARγ expression, we performed Western blot analysis using a PPARγ-specific antibody. We observed the level of nuclear PPARγ protein to decrease with age and to be altered after LPS treatment (Figure 4C). When comparing old and young rats in the control group, we found that the nuclear PPARγ protein level was significantly reduced (49%) in old rats compared with young rats. However, in LPS-treated groups, old rats showed an even greater reduction of nuclear PPARγ level compared with LPS-treated young rats. The old LPS-treated rats expressed the lowest amount of nuclear PPARγ proteins among all groups.

**DNA Binding Activity of PPRE During Aging and LPS**

To investigate the binding of PPAR to the PPRE consensus sequence during aging and inflammation, we used EMSA with nuclear extracts prepared from aged rat kidney to confirm PPAR DNA binding activity. EMSA data (Figure 5) showed that the PPAR binding activity decreased in old rats compared with young rats. Moreover, this age-related down-regulation was further decreased by the LPS challenge, indicating an increased susceptibility to inflammatory stimuli with age. This result corresponded with the decreases in nuclear PPARs and gene expression.

**DISCUSSION**

In a previous study, our group showed that age-related inflammation and oxidative stress enhanced various mRNA
levels and protein expression in aged rat kidney (13). Although many studies have shown a close association between redox-regulated transcription factors and aging and/or inflammation (10), the effects of age on the alterations of PPARs are not well explored. Recent molecular data reveal that the susceptibility of aged animals to inflammatory insults may be due to an increased oxidative state and a disrupted redox state in these animals (13,24).

To better understand the alterations of PPARs during aging and their modulation at the molecular level, we assessed the extent of PPAR changes in mRNA and protein levels with age. In addition, we injected inflammatory LPS into young and old rats to examine a possibly altered PPAR status by prooxidative inflammation. Results revealed an age-related reduction of PPARα and PPARγ in both mRNA and nuclear protein levels, which was detected in LPS-treated rats. Activation of PPARs is indicated by DNA-binding to PPRE, the PPAR binding domain of the transcriptional regulating region. Our data provided the first direct evidence of altered PPARs with age and decreased PPAR DNA binding by inflammation. More interestingly, our results showed for the first time that the antioxidative effects of CR blunt age-induced decreases in PPARα and PPARγ mRNA levels and protein expression. Data further revealed that CR improved the age-induced decrease in PPAR DNA binding activity to PPRE.

The down-regulation of PPARs by inflammation and oxidative stress was reported previously (22,30). Considering
MODULATION OF PPAR IN AGING, INFLAMMATION, AND CR

A

Control LPS-treated
Young Old Young Old

PPARγ

GAPDH

B

Control LPS-treated
Young Old Young Old

PPARα

GAPDH

C

Control LPS-treated
Young Old Young Old

PPARγ

GAPDH

![Graphs showing modulation of PPAR in aging, inflammation, and CR.](https://academic.oup.com/biomedgerontology/article-abstract/59/10/B997/667884)
the close relation between oxidative stress and aging, the possible influence of aging on PPARs alterations can be expected. Among the altered expressions of inflammatory mediators, NF-\(\kappa\)B and its related genes are of importance (13). Chung and colleagues recently proposed the molecular inflammatory hypothesis of aging to emphasize the organism’s susceptibility to incessant oxidative stresses and a disrupted redox balance (at submicroscopic and molecular levels), which gradually lead toward a proinflammatory stage during aging, which may develop into a full-blown pathologic condition during senescence (13).

So far, there are just a few studies reporting the age effect on PPAR expression, which focused mainly on PPAR\(\alpha\). For instance, Chao and colleagues (31) reported that aging had no effect on PPAR\(\alpha\) expression but that RXR changed in liver. However, these authors’ findings conflict with those by Iemitsu and colleagues (21) and Carrera and colleagues (32), who showed that age causes a marked reduction in the expression and activity of PPAR\(\alpha\) in old rat liver and heart. Our current data obtained from the kidney agree with what was found about old rat liver and heart.

Recently, a report (22) on an animal model of aging showed that the supplementation of a PPAR\(\alpha\) agonist, Wy 14,643, and dehydroepiandrosterone (DHEAS) corrected the age-induced up-regulation of NF-\(\kappa\)B activity and the expression of several NF-\(\kappa\)B-regulated genes. In addition, PPAR\(\alpha\)-deficient mice are shown to have higher oxidative stress at an earlier age than wild-type mice and have an exacerbated inflammatory response to LPS stimulation (33). Taken together, these results give a strong implication to PPAR\(\alpha\) in the inflammatory process and oxidative stress. PPAR\(\gamma\), another member of the PPAR subfamily, may also be involved in modulating inflammation. PPAR\(\gamma\) activation through its agonists could interfere with the NF-\(\kappa\)B and AP-1 pathways, and down-regulate NF-\(\kappa\)B and AP-1-dependent genes, including COX-2, iNOS, and cytokines. However, the molecular mechanisms by which PPAR\(\gamma\) and PPAR\(\alpha\) regulate inflammatory response genes are not fully understood. A recent paper reports the activation of PPAR\(\alpha\)-induced I\(\kappa\)B\(\alpha\) mRNA and protein expression and reduced NF-\(\kappa\)B DNA binding activity, although it did not affect p65 nuclear translocation (34). PPAR\(\gamma\) inhibits NF-\(\kappa\)B-driven transcription by physically interacting with both p65 and p50 (35).

Much of the accumulated evidence strongly implicates oxidative stress as a major factor contributing to the aging process and age-related diseases, such as neurodegenerative diseases, arteriosclerosis, diabetes, and inflammatory diseases (10). Recent molecular studies show that the induction of oxidative stress is associated with the down-regulation of

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**Figure 5. Effects of age and lipopolysaccharide (LPS) on the PPAR-response elements (PPRE) DNA binding activity.** A, The electrophoretic mobility shift assay (EMSA) method was used to compare nuclear peroxisome proliferator-activated receptor (PPAR) binding activities in kidney nuclear protein. Lane 1: probe without nuclear protein sample (BL). Lane 2–5: kidney nuclear protein samples of control (CTL) and LPS-treated (LPS) rats (\(n = 6\) per group). Lane 6: competition assay using 100-fold excess of unlabeled PPRE oligonucleotide. A representative result is presented from three experiments. B, Densitometric measurements showed age and LPS-effected PPAR-DNA binding activity in rat kidneys. The data shown are presented as young LPS untreated rat. Statistical significance: *\(p < .05\); **\(p < .01\) versus young untreated rats; #\(p < .05\); ##\(p < .01\) LPS-treated rats versus the age-matched untreated rats.
PPARs (36–38) and that activation of PPARα leads to a neuroprotective effect (39). In addition, the activation of PPARγ is shown to reduce oxidative stress and nitritative stress (40).

Our findings on age-induced inflammation of PPARs were further tested in LPS-challenged rats. As expected, we observed that aged animals showed a much greater responsiveness to the LPS challenge than the young, implying an enhanced susceptibility and sensitivity to the inflammatory stimulus with age. Thus, the LPS challenge exacerbated the already existing oxidative stress (41,42), which may lead to further decreases in PPARs.

The possible inverse association between age-related changes in PPARs levels and oxidative stress was further supported by the antioxidative action of CR. Recent studies on CR provide clear evidence that CR likely exerts its antioxidative and antiinflammation action.

**Conclusion**

Our current data showed that the down-regulation of PPARs by the aging process might be correlated with age-related oxidative stress, which was counteracted by the antioxidative action of CR. Based on these and other data, we propose that the prevention of the age-related decline in antioxidative action of CR, based on these and other data, we propose that the prevention of the age-related decline in antioxidative action of CR.

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