JOURNAL OF MEDICINAL FOOD
J Med Food 12 (2) 2009, 345–350

DOI: 10.1089/jmf.2007.0660

Peroxisome Proliferator-Activated Receptor Activation by a Short-Term Feeding of Zingerone in Aged Rats

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ABSTRACT Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor family, are key regulators of various metabolic pathways related to lipid and glucose metabolism as well as inflammation. We examined the effect of zingerone, a major ingredient of ginger, on PPAR, hepatic nuclear factor-4 (HNF-4), and nuclear factor-κB (NF-κB) expression in 21-month-old male Sprague-Dawley rats. Two experimental groups receiving doses of either 2 or 8 mg/kg/day zingerone for 10 days were compared with young rats (6 months old) and an age-matched control group. For molecular work, the endothelial cell line YPEN-1 was used. Both the 2 and 8 mg/kg/day dose of zingerone significantly increased DNA binding activities of PPARs (2.8-fold). Expression of HNF-4 was also increased in the group receiving the 8 mg/kg/day dose. We further showed that zingerone partially prevented the age-related decline in PPAR expression. *In vitro* experiments revealed zingerone (10 μ*M*) increased PPAR expression (2.5-fold) to a similar extent as the PPAR agonist fibrate (5 μ*M*) and suppressed pro-inflammatory transcription factor NF-κB activity. Collectively, our findings suggest that zingerone exerts its potent anti-inflammatory action by increasing HNF-4 and PPAR activities, while suppressing NF-κB activity.

KEY WORDS: • ***aging*** • ***hepatic nuclear factor-4*** • ***inflammation*** • ***peroxisome proliferator-activated receptor*** • ***zingerone***

INTRODUCTION

DURING THE AGING PROCESS, a progressive decline in function and host defense occurs over time, which increases vulnerability to many inflammatory-related diseases.[1] Emerging evidence indicates that many disease conditions are associated with activated pro-inflammatory transcription factors and altered gene expression.[2,3] Inflammation is an essential immunologic protective mechanism involved in repair of damaged tissue and recovery from infection, which has been shown to be dysregulated during aging.[4,5]

Recent findings indicate a number of major molecular alterations occur during aging that lead to reduced mRNA levels, nuclear protein levels, and DNA binding activity of peroxisome proliferator-activated receptors (PPARs) in the kidney.[6] PPARs are transcription factors belonging to the nuclear hormone receptor family, which can be detected in various species with tissue-specific expression.[6,7] PPARs have a wide array of biological effects, including increasing cell proliferation, glucose and lipid metabolism, insulin sensitivity, and tissue remodeling. Recent studies reveal that these nuclear hormone receptors also can regulate the expression of inflammatory genes, such as cytokines, metalloproteases, and acute-phase protein by modulating the inflammatory mediator nuclear factor-κB (NF-κB).[8–10] Therefore, modulation of activity and expression of PPARs may be a potential molecular intervention to reduce age-related vulnerability to inflammatory disease.

Although many natural substances have been found to have anti-inflammatory effects, their molecular actions are not well characterized. Zingerone is a pungent pyrolytic product of ginger that has been found to have potent antioxidant,[11] anticancer,[12] and anti-inflammatory[13] effects. The anti-inflammatory properties of zingerone appear to be related to the suppression of the inflammatory actions of macrophages and release of monocyte chemoattractant protein-1 from adipocytes.[13] We recently found zingerone can suppress age-related NF-κB activation and several of its tar-

Manuscript received 11 November 2007. Revision accepted 17 February 2008.

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get genes, like cyclooxygenase-2 and inducible nitric oxide synthase in senescent male rats (authors' unpublished data). However, to date, the molecular actions underlying the effect of dietary zingerone on modulation of expression of PPARs has not been reported. The chemical structure of zingerone is shown in Figure 1A. In the present study, we examined the effect of short-term doses of zingerone on expression of PPARs, nuclear protein levels, NF-κB, and hepatic nuclear factor-4 (HNF-4).

MATERIALS AND METHODS

Materials

Unless otherwise stated, all compounds were obtained from Sigma Chemical Co. (St. Louis, MO). Zingerone was purchased from Aldrich Chemical Co. (Milwaukee, WI). PPARα, PPARβ, and PPARγ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Immobilon-P transfer membrane was obtained from Millipore Corp. (Bedford, MA). HNF-4 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) western blot detection reagents were purchased from Amersham Life Science, Inc. (Arlington Heights, IL).

Animal experiments

Male Sprague-Dawley rats (21 months old) (Samtako, Osan, Republic of Korea) were used for all studies. Rats were fed a diet of the following composition: 21% soy bean 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 (International Fiber Corp., North Tonawanda, NY). Animals were housed and handled in a controlled environment (24°C; 50–60% humidified atmosphere) according to guidelines of the Animal Care Committee of the Pusan National University, Busan, Republic of Korea.

Rats at 6 and 21 months of age were grouped as young and old, respectively. Zingerone was mixed with powder and fed to the 21-month-old rats at a dose of 2 or 8 mg/kg/day.

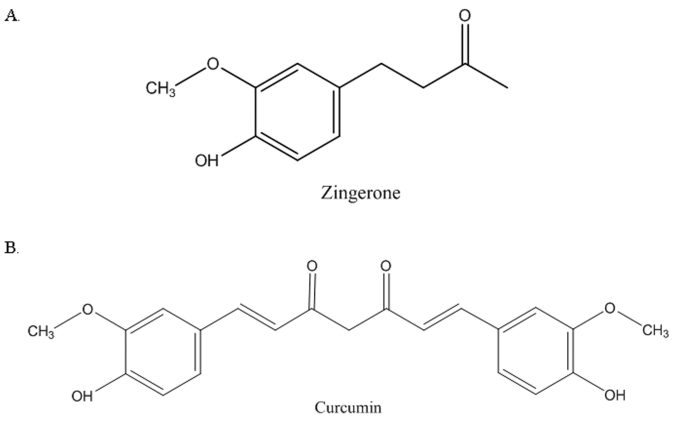

FIG. 1. Chemical structure of (**A**) zingerone and (**B**) curcumin.

The two experimental groups were compared with an age-matched control group. Each group contained five rats. After 10 days of feeding, the rats were sacrificed by decapitation, and kidneys were quickly removed and rinsed in ice-cold buffer (100 m*M* Tris, 1 m*M* EDTA, 0.2 *M* phenylmethylsulfonyl fluoride, 1 μM pepstatin, 2 μM leupeptin, 80 mg/L trypsin inhibitor, 20 m*M* β-glycerophosphate, 20 m*M* NaF, and 2 m*M* sodium orthovanadate, pH 7.4). The kidney tissue was immediately frozen in liquid nitrogen and stored at −80°C until later used for the current study. The kidney was used for the study because of its vulnerability to inflammation and its sensitivity to the anti-inflammatory response.

Cell cultures

The rat prostate endothelial cell line YPEN-1 was obtained from American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan) containing 2 m*M* L-glutamine, 100 mg/mL streptomycin, 2.5 mg/L amphotericin B, and 5% heat-inactivated fetal bovine serum. Cells at exponential phase were used for all experiments.

Protein and western blot analyses

The protein concentration was determined by the BCA method (Sigma) using bovine serum albumin as a standard. Homogenized samples were boiled for 5 minutes with a gel-loading buffer (pH 6.8) composed with 0.125 *M* Tris-Cl, 4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, and 0.2% bromophenyl blue in a 1:1 ratio. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 6–17% acrylamide gels. The gels were subsequently transferred onto a nitrocellulose membrane (Hybond C, Amersham). The membrane was immediately placed in a blocking solution (5% nonfat dry milk in TBS-T buffer [10 m*M* Tris, 100 m*M* NaCl, and 0.1% Tween 20, pH 7.5]) at room temperature for 1 hour. The membrane was washed in TBS-T buffer for 30 minutes and then incubated with the first antibody at room temperature for 2 hours. After three 10-minute washings in the TBS-T buffer, the membrane was incubated with a second antibody at room temperature for 1 hour. After four 10-minute washings in the TBS-T buffer, antibody labeling was detected using ECL following the manufacturer's instructions and then exposed to radiographic film. Prestained blue protein markers were used for molecular weight determinations.[14]

Preparation of nuclear extracts

Nuclear extracts were prepared, and assays were performed by modified procedures as previously described by Wang *et al.*[15] In brief, cells were harvested, washed twice with phosphate-buffered saline, and then subjected to centrifugation at 3,000 *g* for 5 minutes at 4°C. The washed cells were resuspended in hypotonic buffer containing 10 m*M* Tris-HCl (pH 8.0), 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 0.1% Nonidet P40, 5 μg/mL pepstatin, 5 μg/mL aprotinin,

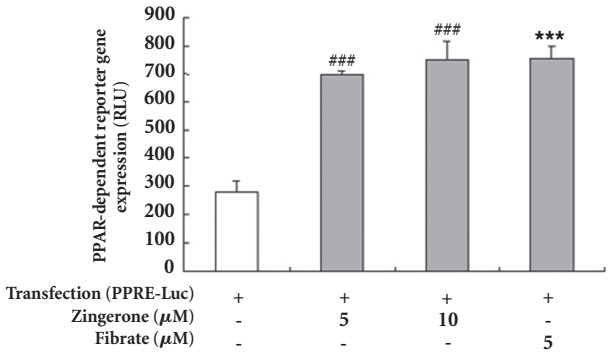

FIG. 2. Modulation of PPAR activity by zingerone in YPEN-1 cells. Cells were grown to 50–60% confluence after transfection of a reporter plasmid. The cells were incubated in serum-free medium with zingerone and fibrate for 6 hours. Luc, luciferase; RLU, relative luminescence units. Statistical significance: $^{###}P < .001$ for 5 μM zingerone versus 10 μM zingerone; $^{***}P < .001$ compared to untreated with zingerone.

and 10 μM *N*-CBZ-Leu-Leu-Leu-Ala (Sigma) and stood on ice for 20 minutes. Then these cells were subjected to centrifugation at 12,000 *g* for 15 minutes at 4°C. The pellets were resuspended in nuclear extract buffer (10 m*M* Tris-HCl [pH 8.0], 50 m*M* KCl, 300 m*M* NaCl, 1 m*M* dithiothreitol, 5 μg/mL pepstatin, 5 μg/mL aprotinin, and 10 μM *N*-CBZ-Leu-Leu-Leu-Ala). After incubating on ice for 30 minutes, the samples were subjected to centrifugation at 12,000 *g* for 30 minutes at 4°C. The supernatant was collected directly and defined as the nuclear extract.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed according to the manufacturer's instructions. The binding reaction was optimized in binding buffer containing 10 m*M* HEPES, 0.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 80 m*M* KCl, and 100% glycerol with PPAR oligonucleotide 5'-TGACCTTTGACCTAGTTTTG-3' (Santa Cruz Biotechnology) for 20 minutes at room temperature. Protein–DNA binding assays were performed with nuclear extracts (25 μg). The DNA–protein complexes were separated from unbound oligonucleotides on a native 4% gradient polyacrylamide gel at 200 V in TBE buffer (50 m*M* Tris [pH 8.0], 45 m*M* borate, and 0.5 m*M* EDTA). After separation was achieved, the gel was vacuum-dried for autoradiography and exposed to Fuji radiographic film (Fuji Photo Film Co., Tokyo).

Transient transfection and luciferase reporter assays

For the luciferase assays, 0.1 μg of plasmid was transfected to 2×10^4 YPEN-1 cells per 48-well plate in 500 μL of Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum at 37°C under a humidified atmosphere of 95% air/5% CO_2. Cells were transfected with FuGENE 6 transfection reagent (Roche, Indianapolis, IN), and plasmids were used for transfection with the 3×AOX-TK-luciferase reporter vector (University of California at San Diego, La Jolla, CA) and pTAL-NF-κB (Clontech, Mountainview, CA). The cells were treated with zingerone and fibrate and then detected with Steady-Glo® luciferase assay system (Promega, Madison, WI). Luciferase activity was measured by a luminometer (GENios, TECAN, Schweiz AG, Mannedorf, Switzerland).

Statistical analysis

One-way analysis of variance and paired *t* tests were conducted to examine between-group differences. All statistical tests were two tailed, and significance was accepted at $P = .05$. Data are presented as mean ± SE values at each time point.

RESULTS

Increased PPAR activity by zingerone in YPEN-1 cells

The 3×AOK-TK-luciferase reporter constructs with three copies of the PPAR response element (PPRE) were transiently transfected to YPEN-1 cells, followed by stimulation with increasing amounts of zingerone (5 or 10 μM). These data showed that treatment with zingerone at 10 μM led to a 2.5-fold increase compared to untreated cells (Fig. 2). The PPAR agonist fibrate (5 μM) was applied as a positive control to monitor the activation of luciferase reporter, and it showed an increase in PPRE activity (2.5-fold). These results suggest that zingerone induced activity of PPAR activity in YPEN-1 cells.

Increased nuclear PPAR protein levels and binding activity in zingerone-fed old rats

In order to determine the effects of zingerone feeding on age-related alterations of PPARs protein levels, a western blot analysis was performed with antibodies specific for PPARα, PPARβ, and PPARγ proteins in rat renal nuclear extracts. Results indicated that the oral administration of zingerone to 21-month-old male rats partially prevented the age-related decrease in nuclear PPARα, PPARβ, and PPARγ. As compared with values obtained in age-matched controls, the 2 mg/kg/day dose of zingerone increased nuclear PPARα (1.5-fold), PPARβ (2.7-fold), and PPARγ (1.1-fold), and the 8 mg/kg/day dose of zingerone increased expression of PPARα (1.9-fold), PPARβ (2.2-fold), and PPARγ (1.7-fold) (Fig. 3).

In untreated rats, incubation of rat renal nuclear extracts with a PPRE oligonucleotide yielded a single, PPAR-specific band that decreased PPAR DNA binding activity (2.4-fold) in nuclear extract in old rats compared to young rats. Nuclear extracts from zingerone-treated old rats, however, showed a 2.8-fold increase in the intensity of the specific band corresponding to the binding activity of PPARs (Fig. 4). Taken together, the above results demonstrate that zingerone plays a role in enhanced DNA binding and consequently for transactivation of PPARs.

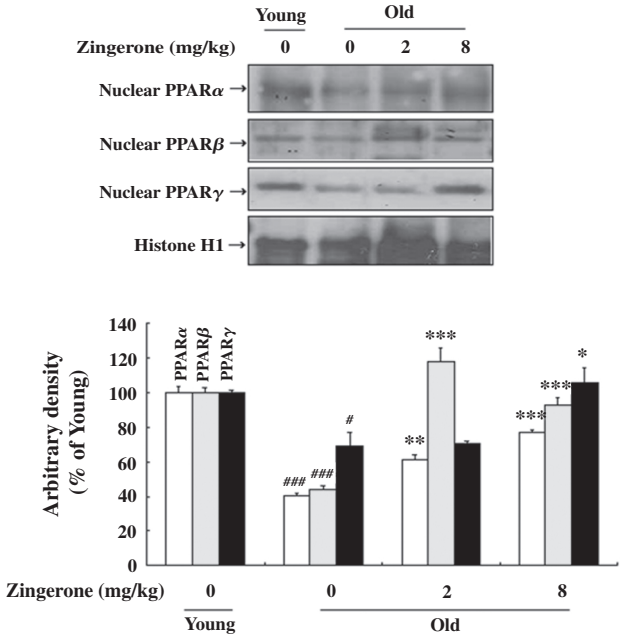

FIG. 3. Effects of zingerone on renal expression and activity of PPARs in old male rats. Western blot analysis was performed to detect PPAR$\alpha/\beta/\gamma$ protein levels in nuclear extract from young and old rats and old rats fed zingerone. Statistical significance: $^{\#}P < .05$, $^{\#\#\#}P < .001$ versus young rats; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus zingerone-untreated old rats.

Modulation of HNF-4 content by zingerone feeding

Transcription factors (HNF-4) participate in the transcriptional regulation of the PPAR by increasing its gene expression.[16] To assess whether HNF-4 is age-dependent or not, we analyzed the nuclear HNF-4 protein in renal tissues. As shown in Figure 5, HNF-4 protein content was reduced by 42% in the kidney of old rats, which was counteracted (18%) by zingerone supplementation (Fig. 5).

Down-regulation of NF-κB activation by zingerone

To verify whether zingerone modulates NF-κB activity or not, pTAL-NF-κB luciferase reporter vector was transiently transfected in YPEN-1 cells. Cells were stimulated with zingerone (20 μM) for 2 hours. Then, 100 μM *t*-butylhydroperoxide (*t*-BHP) was treated and incubated for 6 hours. The relative luciferase activity was increased after the *t*-BHP treatment. Zingerone significantly suppressed increased luciferase activity ($P < .05$; Fig. 6). The PPAR agonist fibrate (20 μM) was applied as a negative control to monitor the activation of luciferase reporter, and it showed a decrease in NF-κB activity. Thus, these results suggest that NF-κB activity was suppressed by zingerone in YPEN-1 cells.

DISCUSSION

Age-related decreases in PPAR content and activity were reported to display a set of phenotypic characteristics related to lipid and glucose metabolism that are common in individuals with the metabolic syndrome.[17,18] Emerging evidence strongly links the molecular inflammatory process as a major underlying cause for many age-related diseases.[5] As previously reported by Sung *et al*.,[6] the molecular modulation of PPARs may be a key mechanism by which calorie restriction exerts its anti-inflammatory and anti-aging effects. Similar to calorie restriction, our results indicate that administration of zingerone can reverse the age-related reduction of nuclear PPAR and the activated NF-κB.

Our present data show that zingerone led to an increase in PPARs in YPEN-1, suggesting that zingerone may act as a PPAR agonist. In support of this, DNA binding activity of PPARs was found to be increased in the kidney tissues of zingerone-fed rats. Previous studies have reported that several other natural products exert their anti-inflammatory effect through activation of PPARs. For instance, Choi *et al*.[9] reported that 3-methyl-1,2-cyclopentanedione from coffee extract suppresses the NF-κB activity through PPARγ activation in the endothelial cell line YPEN-1. Also, it is worth noting that curcumin, the principal curcuminoid of curry spice turmeric, exerts its anti-inflammatory effect by up-regulating PPAR.[19,20] Interestingly, curcumin is composed of the dimer structure of zingerone, providing the structural basis of zingerone's efficacy (see Fig. 1B). Although not tested, we speculate that zingerone's carboxylate group through chemical shifts may be responsible for the

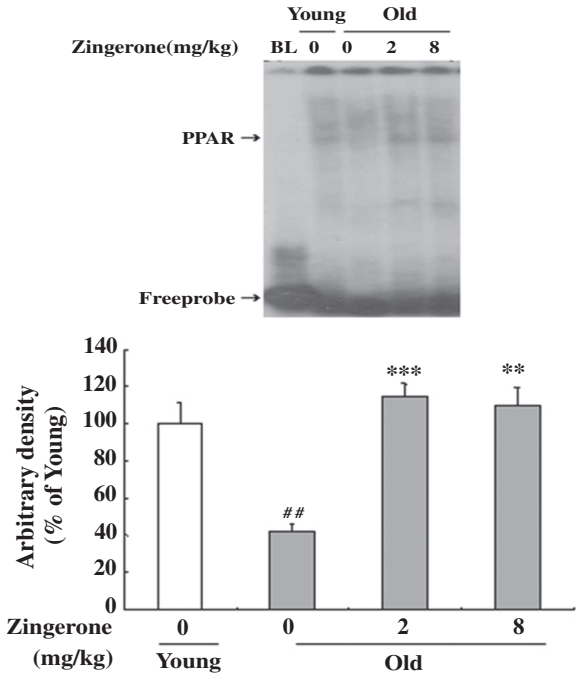

FIG. 4. Effect of zingerone on age-related PPAR DNA binding activity. Young rats (6 months old) and old rats (21 months old) were used. Zingerone (2 or 8 mg/kg/day) was administered to the old group for 10 days. BL, blank. Statistical significance: $^{\#\#}P < .01$ versus young rats; $^{**}P < .01$, $^{***}P < .001$ versus zingerone-untreated old rats.

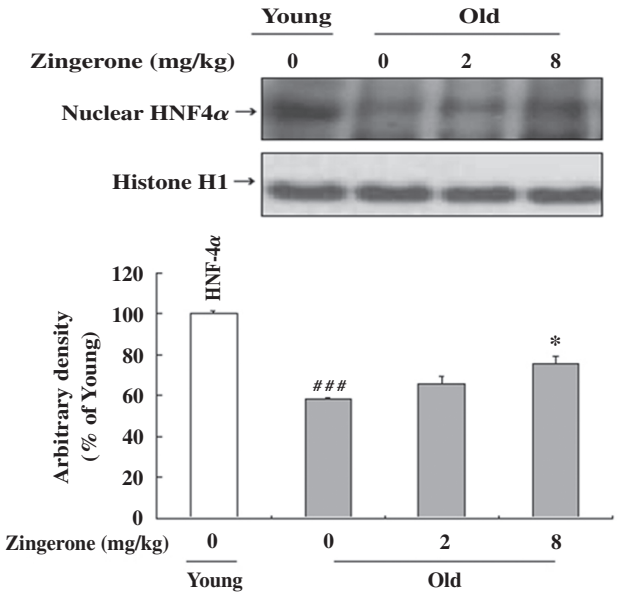

FIG. 5. Effect of zingerone on age-related nuclear HNF-4. Nuclear extracts were prepared from kidney of young and old rats and old rats fed zingerone. Western blot analysis was performed to detect HNF-4 protein levels. Statistical significance: $^{###}P < .001$ versus young rats; $^{*}P < .01$ versus zingerone-untreated old rats.

hydrogen bond with a residue of the AF2 helix in PPAR molecules as the PPAR agonist.

NF-κB is a transcription factor responsible for regulating the transcription of several major genes involved in the inflammatory process, which are critical to generating a proper immune response to infection and injury. The molecular exploration performed in our previous study provides evidence that NF-κB activation plays a key role in the induction of

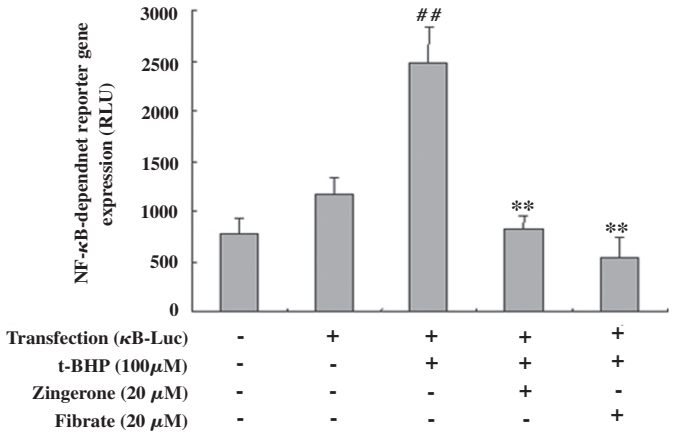

FIG. 6. Inhibition of NF-κB activity by zingerone in YPEN-1 cells. Cells were permanently transfected with an NF-κB-containing plasmid linked to the luciferase gene, and then cells were co-treated with *t*-BHP for 6 hours after preincubation with zingerone (20 μM) and fibrate (20 μM) for 2 hours. RLU, relative luminescence units. Statistical significance: $^{##}P < .001$ versus untreated transfection control; $^{**}P < .01$ versus 100 μM *t*-BHP-treated group.

the inflammatory process during aging.[4,21–23] Several lines of evidence indicate that PPAR activation by its agonists inhibits the NF-κB pathway, thereby regulating inflammatory gene expression.[24,25] For example, we demonstrated in previous study that the PPAR agonist 2,4-thiazolidinedione is capable of regulating NF-κB activation and its target genes during aging.[26] In agreement with these findings, our present data show that zingerone treatment attenuates NF-κB activation as determined by the luciferase reporter assay.

Our finding that HNF-4 expression is increased following supplementation with zingerone reveals a potential pathway by which zingerone may lead to PPAR activation (Fig. 5). It has been reported that transactivation of PPARs is modulated by HNF-4 because the PPAR promoter contains several DR-1-HNF-4 response elements.[27,28] Therefore, our findings suggest that the elevated nuclear HNF-4 expression following zingerone supplementation may lead to increased expression of PPARs.

In conclusion, our study showed that a short-term feeding of zingerone to senescent male rats suppresses the age-related reduction in nuclear PPAR level and activity. The increased nuclear HNF-4 is speculated to be the factor responsible for the up-regulation of PPARs by zingerone. We further speculate that zingerone can have wide therapeutic applications for inflammation-related diseases.

ACKNOWLEDGMENTS

This study was supported by the Technology Development Program (No. 105075-3) for Agriculture and Forestry, Ministry of Agriculture and Forestry, and a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean Government (MOST) (number 2007-00376), Republic of Korea. This study was financially supported by Pusan National University in the 2007 Post-Doctoral Program. We are grateful to the "Aging Tissue Bank" for supplying aged tissue. The authors are grateful to Dr. Christopher K. Glass (University of California at San Diego) for his kind gift of reporter plasmid construct.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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