Peroxisome Proliferator-activated Receptor α Activation Modulates Cellular Redox Status, Represses Nuclear Factor-κB Signaling, and Reduces Inflammatory Cytokine Production in Aging*

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In aged mice, the redox-regulated transcription factor nuclear factor-κB (NF-κB) becomes constitutively active in many tissues, as well as in cells of the hematopoietic system. This oxidative stress-induced activity promotes the production of a number of pro-inflammatory cytokines, which can contribute to the pathology of many disease states associated with aging. The administration to aged mice of agents capable of activating the α isoform of the peroxisome proliferator-activated receptor (PPARα) was found to restore the cellular redox balance, evidenced by a lowering of tissue lipid peroxidation, an elimination of constitutively active NF-κB, and a loss in spontaneous inflammatory cytokine production. Aged animals bearing a null mutation in PPARα failed to elicit these changes following treatment with PPARα activators, but remained responsive to vitamin E supplementation. Aged C57BL/6 mice were found to express reduced transcript levels of PPARα and the peroxisome-associated genes acyl-CoA oxidase and catalase. Supplementation of these aged mice with PPARα activators or with vitamin E caused elevations in these transcripts to levels seen in young animals. Our results suggest that PPARα and the genes under its control play a role in the evolution of oxidative stress excesses observed in aging.

Multicellular organisms have evolved complex homeostatic mechanisms to sense and respond to a diverse range of exogenous and endogenous signals. One such mechanism appears to require the biochemical events which follow the activation of a peroxisome proliferator-activated receptor (PPAR). PPARs are members of the nuclear steroid hormone receptor superfamily and function to transduce a variety of environmental, nutritional, and inflammatory signals into a defined set of cellular responses (1). Three PPAR isoforms, α, β/δ, and γ, have been identified (1–3). Each exhibits a high degree of sequence and structural homology (2), but they possess individual patterns of tissue distribution (4, 5). Activation of a PPAR causes the receptor to heterodimerize with a 9-cis-retinoic acid receptor (6), thereby conferring upon it the ability to bind DNA and transcriptionally regulate a subset of genes possessing a peroxisome proliferator response element, consensus 5′-AAGTC-TGCAAGGTTCA-3′ (7), in their promoter region (1, 8, 9).

While PPARs had long been considered to be orphan receptors, recent reports indicate that a number of natural and xenobiotic ligands with specificity for PPARα do indeed exist. The first ligands described were the insulin-sensitizing thiazolidinediones which are specific ligands for the PPARγ isofrom (10). Since that time, a number of natural endogenous molecules have been found to be capable of activating PPARs. For example, 15-deoxy-Δ12,14-prostaglandin J2 represents a natural PPARγ ligand (11, 12). Many specific fatty acid species and their derivatives, especially polyunsaturated fatty acids (13–16), the inflammatory mediator leukotriene B4 (17), and the eicosanoid 8(S)-hydroxyeicosatetraenoic acid (18), have now been demonstrated to be ligands for PPARα. Studies employing the PPARα-deficient mouse have also revealed that the natural steroid hormone dehydroepiandrosterone-3β-sulfate (DHEAS) is a specific activator of PPARα (19). Furthermore, a vast array of man-made compounds are capable of activating PPARs. These include the synthetic arachidonic acid analog eicosatetrayenoic acid, the hypolipidemic agents WY-14,643 and clofibrate, certain non-steroidal anti-inflammatory drugs, phthalate ester plasticizers, plus a number of other xenobiotic compounds (1, 20, 21). Based upon their capacity to elicit cellular responses to a variety of stimuli, the PPARs may represent a class of molecules which allow the biochemical adaptation to a diverse range of internal and external signals. These include nutritional and inflammatory agents as well as a number of potentially toxic substances. PPARα activation results in the transcriptional up-regulation of many genes, including those involved in peroxisomal and mitochondrial fatty acid β-oxidation, some lipid binding proteins and apolipoproteins, certain isozymes of the cytochrome P450 family, and antioxidant enzymes (1, 17, 22, 23). In addition, activation of PPARs has been demonstrated to antagonize signaling through an array of important pathways, including STATs, AP-1, and NF-κB (13, 24–28).

There is strong evidence to suggest that the deleterious changes to the immune system that occur as an individual or experimental animal ages, including a reduced capacity to be effectively vaccinated and the dysregulated production of a number of pleiotropic cytokines, are associated with a decreased ability to effectively handle oxidative stress (29–33). That elevated levels of cellular oxidative stress are present in...
aged experimental animals is indicated by elevations in tissue and circulating lipid peroxide levels (32–34) as well as oxidized proteins (35, 36). The redox-regulated and oxidant stress-activated transcription factor NF-κB has been reported to be active in the heart, liver, kidney, brain, and cardiac muscle of aged experimental animals, without alterations in the amounts of NF-κB subunits or of the inhibitory molecule IκBα present in the cytosol (37–39). Under resting conditions, NF-κB exists in the cytoplasm as a dimer bound to the inhibitory protein IκB. Signaling by various cell stimuli appear to converge at the generation of increased levels of intracellular reactive oxygen species (ROS), causing the phosphorylation and ubiquitination of IκB and its release from NF-κB. The NF-κB dimer then translocates to the nucleus and binds to the promoter region of genes possessing a κB motif (consensus GGGRRNT(Y/C)), thereby causing recruitment of transcriptional machinery and induction of gene transcription (40).

We have recently demonstrated that NF-κB is also present in an active state in the macrophages and lymphocytes which reside in the spleens of aged mice (34). This active NF-κB was demonstrated to correlate with the expression of the NF-κB-regulated genes IL-6, IL-12, macrophage migration inhibitory factor, cyclooxygenase-2, and tumor necrosis factor-α (34). We found that the administration of specific PPARα activators or the dietary antioxidant vitamin E to aged rodents effectively reduced the elevated levels of active NF-κB, reestablished control over proinflammatory cytokine production, and reduced lipid peroxide levels in various tissues (34). These findings suggest a role for PPARα in the maintenance of redox balance during the aging process.

The studies presented herein demonstrate that low dose DHEAS or WY-14,643 administration to aged animals elicits a number of biologic changes, which are mediated through a process involving PPARα activation. We employed a strain of mice bearing a null mutation in PPARα (PPARα–/–) to experimentally demonstrate that normal PPARα function is necessary to effectively maintain a balance in cellular redox state. PPARα–/– mice were found to express indicators of oxidative stress much earlier in their lifespans than wild-type mice. Administration of PPARα-specific activators to wild-type and knockout animals was capable of reducing the age-associated elevations in NF-κB activity and constitutive pro-inflammatory cytokine production in the PPARα wild-type animals but not their PPARα-deficient counterparts. Vitamin E supplementation lowered the levels of active NF-κB present in the spleens of both aged wild-type and aged knockout animals. Similar results were obtained using young PPARα+/+ and PPARα–/– mice rendered redox-imbalanced at an early age by feeding a vitamin E-deficient diet. In addition, a decline in cellular PPARα expression was observed to occur with normal aging. This was accompanied by similarly reduced levels of acyl-CoA oxidase and catalase mRNA expression, enzymes that are transcriptionally up-regulated following administration of PPARα activators. Either vitamin E supplementation or treatment of aged mice with PPARα activators was able to cause elevations in splenic PPARα, acyl-CoA oxidase, and catalase mRNA to levels typically observed in the spleens of young animals. Our findings indicate that the therapeutic administration of PPARα activators is able to regulate cellular antioxidant/antioxidant balance through a mechanism that appears to require a functional PPARα.

**EXPERIMENTAL PROCEDURES**

**Animals and Diets—**Colonies of PPARα wild type (PPARα+/-) and homozygous knockout (PPARα–/–) mice were expanded from breeding pairs obtained from Dr. F. J. Gonzalez (Metabolism Branch, National Institutes of Health, Bethesda, MD). The derivation and phenotypic characteristics of these animals have previously been reported (41). Female mice were used for all of the experiments reported herein. Six-week-old and 20–24-month-old female C57BL/6 mice were purchased from the National Institute on Aging. All mice were housed in the University of Utah Animal Resource Center, which routinely monitors animals for the most prevalent murine pathogens, employs sentinel animals as a means for early detection of murine hepatitis virus, and is subject to strict compliance with regulations established by the Animal Welfare Act. Normal maintenance chow, vitamin E-deficient (tocopherol-stripped) chow, and vitamin E-containing control chow were purchased from Harlan Teklad (Madison, WI). Mice were anesthetized with halothane and sacrificed by cervical dislocation. Any mice with evidence of gross internal or external pathology at the time of sacrifice were excluded from the study.

**Supplementation Therapy—**DHEAS (5-androsten-3β-ol-17-one 3-sulfate) (Sigma), dissolved directly in the drinking water at a concentration of 500 μg/ml, was prepared fresh on a weekly basis and was available to the mice ad libitum, resulting in doses of approximately 300–500 μg/day. WY-14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid) (Chemsys Science Laboratories, Lenexa, KS) was added directly to the chow resulting in 250 μg/day doses. α-Tocopherol (Sigma) was applied to the chow in order to achieve a supplemental vitamin E dose of 4.6 mg/day. Peroxisome-proliferating doses of DHEA (5-androsten-3β-ol-17-one) (Sigma) and WY-14,643 were provided in the chow at 0.5% w/w (25 mg/day) and 0.1% w/w (5 mg/day), respectively. Freshly prepared food was provided every 2–3 days and was continuously available to the mice during the 2-week supplementation period.

**Preparation of Nuclear Extracts—**Nuclear extracts were prepared from cell suspensions of whole spleens using a modified protocol of Dignam et al. (42). Briefly, cells were washed twice in ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in 1 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 10 μg/ml aprotonin, 100 μM leupeptin, 1 mM PMSF, 1 mM DTT, and 0.5% Nonidet P-40) for 10 min on ice, vortexing every 2 min. Nuclei were collected by centrifugation at 1000 × g for 5 min at 4 °C. The nuclear pellet was washed with 1 ml of buffer A without Nonidet P-40. 25 μl of buffer B (10 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/ml aprotonin, 100 μM leupeptin, 1 mM PMSF, and 1 mM DTT) was added to the pellet and, following sonication for 10 s, incubated for 30 min on ice. Nuclear debris was removed by centrifugation at 13,000 × g for 10 min. The supernatant was collected, the protein content of the nuclear extract determined (43), and an electrophoretic mobility shift assay (EMSA) was performed. While contamination of the nuclear extracts with small quantities of cytosolic proteins is possible, cytosolic NF-κB will not bind DNA due to its association with the inhibitory molecule IκBα (44).

**EMSA—**Equal amounts of nuclear extracts (2 μg of protein) were incubated with 30,000 cpm of 32P labeled NF-κB specific probe (Pro-mega). Reactions were performed in a 20-μl total volume containing 2 μg of nuclear extract, 4 μl of 5% gel shift binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA, and 20% glycerol), 1.5 μg of poly(dI-dC), and 1 μl of probe. For supershift assays, 2 μl of an appropriate anti-NF-κB subunit antibody (Santa Cruz Biotechnology, Inc.) was added to each reaction. The reaction was incubated at room temperature for 15 min, loaded on a 4% native polyacrylamide gel, and run in 0.5× TBE buffer. The gel was dried and subjected to autoradiography. NF-κB-specific bands were confirmed by competition with a 50-fold excess of an unlabeled NF-κB probe, which resulted in no shifted band, or by preparing the reaction with excess labeled nonspecific probe, which did not reduce the intensity of the NF-κB band.

**Cell Culture and ELISA—**Cells obtained from the spleens of PPARα+/- or PPARα–/– animals were cultured under serum-free conditions. Briefly, mice were anesthetized with halothane and sacrificed by cervical dislocation. Single cell suspensions were prepared from the spleens of these animals. The collected splenocytes were washed three times in Dulbecco’s phosphate-buffered saline and cultured at 10⁷ cells/ml in freshly prepared serum-free medium consisting of RPMI 1640, 1% Nutridoma-SR (Boehringer Mannheim), 200 μM L-glutamine, antibiotics, and 5 × 10⁻⁵ M 2-mercaptoethanol. Cells were incubated for 24 h at 37 °C in an atmosphere of 5% CO2 in air. Cell culture supernatants were then collected for quantitative evaluation of LPS-stimulated IL-6 or IL-12 (p40) by ELISA, as described previously (45). Mouse peritoneal macrophages were incubated with murine anti-murine cytokine antibodies and murine recombinant IL-6 and IL-12 cytokine standards were purchased from PharMingen (San Diego, CA).
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 RESULTS

PPARα−/− Mice Exhibit an Obese Phenotype—While expanding the PPARα+/+ and PPARα−/− mouse colonies, it became apparent that the body weights of 14-week-old PPARα−/− mice were greater than age-matched PPARα+/+ mice. As has been described previously (48, 49), an observed 30% increase in body weight persisted throughout adulthood, and the overweight phenotype was more exaggerated in males. Gross pathologic examination revealed a markedly increased amount of intraabdominal adipose tissue in the PPARα−/− mice (data not shown). The recent report that PPARα−/− mice demonstrate reduced basal levels of mitochondrial fat-metabolizing enzymes may offer an explanation for these observations (23). An inability to efficiently catabolize fatty acids would cause them to be redirected for storage in adipose tissue, thereby accounting for the obese phenotype of PPARα−/− mice. The reported inability of PPARα−/− mice to transcriptionally up-regulate enzymes for fatty acid catabolism in response to administration of PPARα activators may be why these mice exhibit lipid droplets in their livers following an overnight fast (48, 49), suggesting that endogenous molecules (i.e. fatty acids) are not capable of activating these enzymes, or of being enzymatically metabolized, in the PPARα−/− mice. PPARα−/− Mice Exhibit an Aged Proinflammatory Phenotype at a Younger Chronological Age than PPARα+/+ Mice—When splenocytes were obtained from 4-month-old PPARα+/+ and PPARα−/− mice and analyzed by EMSA for NF-κB activity, PPARα+/+ mice did not express nuclear NF-κB activity in their spleens, while age-matched PPARα−/− mice already exhibited high levels of nuclear NF-κB activity (Fig. 1A). In vitro activation of splenocytes from PPARα+/+ and PPARα−/− mice with a low dose of LPS (10 ng/ml) was able to modestly increase nuclear NF-κB activity in both groups (Fig. 1A). The levels of NF-κB activity seen in splenocytes from 4-month-old PPARα+/+ mice following activation with LPS, however, did not reach the levels seen in splenocytes from PPARα+/+ mice. Splenocytes obtained from 4-month-old PPARα+/+ or PPARα−/− mice were cultured at equivalent cell densities in the absence or presence of 10 ng/ml LPS. After 45 min, cells were harvested, nuclear extracts were prepared, and an NF-κB EMSA was performed (A). Data shown are typical of mice from each group. Cells treated with 0 ng/ml (B) or 10 ng/ml (C) LPS were cultured for 24 h, and IL-6 and IL-12 levels (mean ± S.D. of 3 mice/group) in supernatants were quantitated by ELISA.

FIG. 1. PPARα−/− mice acquire a physiologically aged phenotype, indicative of a state of redox imbalance, at a younger age than PPARα+/+ mice. Splenocytes obtained from 4-month-old PPARα+/+ (open bars) or PPARα−/− (filled bars) mice were cultured at equivalent cell densities in the absence or presence of 10 ng/ml LPS. After 45 min, cells were harvested, nuclear extracts were prepared, and an NF-κB EMSA was performed (A). Data shown are typical of mice from each group. Cells treated with 0 ng/ml (B) or 10 ng/ml (C) LPS were cultured for 24 h, and IL-6 and IL-12 levels (mean ± S.D. of 3 mice/group) in supernatants were quantitated by ELISA.

10 LA). The levels of NF-κB activity seen in splenocytes from 4-month-old PPARα+/+ mice following activation with LPS, however, did not reach the levels seen in splenocytes from age-matched PPARα+/+ mice in the absence of exogenous activators (Fig. 1A). The secretion of two NF-κB-driven cytokines, IL-6 and IL-12, by either control or LPS-activated splenocytes from PPARα+/+ and PPARα−/− mice was quantitated by ELISA. PPARα−/− splenocytes were found to produce 2–3 times more IL-6 and IL-12, both in the absence of exogenous stimulation (Fig. 1B) and following activation with LPS (Fig. 1C), than splenocytes from PPARα+/+ mice.

Peroxisome Proliferating Doses, but Not Therapeutic Doses, of DHEAS or WX-14,643 Cause the Activation of NF-κB in Young PPARα+/+ Mice—We questioned whether the effects that we have previously reported to occur following the administration to aged mice of natural and xenobiotic agents capable of activating PPARα (45, 50)² were dependent upon the presence of a functional PPARα. PPARα+/+ and PPARα−/− mice

DHEAS or WY-14,643 were added to the diets of young PPARα activate NF-κB (Fig. 2). Consistent with their reported inability to respond to peroxisome proliferating doses of DHEAS or WY-14,643 for a period of 2 weeks, spleens were then harvested, nuclear extracts were prepared, and an NF-κB EMSA was performed. Data are representative of results from experiments performed twice employing two mice per group.

at 2 and 15 months of age were compared in these studies. As has been previously demonstrated with C57BL/6 strain mice (45), both PPARα+/+, and PPARα−/− mice at 2 months of age exhibited only minimal levels of nuclear NF-κB activity in their spleens (Fig. 2). When peroxisome-proliferating doses of DHEAS or WY-14,643 were added to the diets of young PPARα+/+ mice for a period of 2 weeks, a marked elevation in nuclear NF-κB activity was consistently observed in spleen (Fig. 2) and liver tissue (data not shown). It is not known whether the induction of NF-κB represents a transient response or is long-lasting and contributes to the hepatocarcinogenic effects of long term and high dose administration of peroxisome proliferators to rodents (52). Spleens of animals provided therapeutic doses of DHEAS or WY-14,643 that have previously been demonstrated by us to cause marked reductions in splenic NF-κB activity, inflammatory cytokine production, and tissue TBARS levels (34), (approximately 40-fold lower than peroxisome proliferating doses) showed no nuclear NF-κB-inducing activity (Fig. 2). Consistent with their reported inability to respond to PPARα activators (41), no nuclear NF-κB was observed in young PPARα−/− mice treated with DHEAS or WY-14,643 at either the high (peroxisome-proliferating) or low doses (Fig. 2). These results demonstrate that, while peroxisome-proliferating doses of PPARα-specific activators are capable of activating NF-κB, lower doses of PPARα activators appear not to cause deleterious changes in cellular redox balance.

Effects Derived from the Therapeutic Administration of DHEAS or WY-14,643 to Aged Mice Are Dependent upon a Functional PPARα—When animals were evaluated at 15 months of age, both PPARα+/+ and PPARα−/− mice were found to abnormally express significant levels of nuclear NF-κB in their unstimulated spleens (Fig. 3A). This observation is consistent with our recent findings in C57BL/6 strain mice (45). When 15-month-old PPARα+/+ and PPARα−/− mice were treated with therapeutic doses (34) of DHEAS or WY-14,643 (300–500 and 250 μg/day, respectively), or with a supplemental dose of the antioxidant vitamin E (3 IU/day), the PPARα+/+ mice exhibited markedly reduced nuclear NF-κB activity in their spleens following all three treatments (Fig. 3A). The levels of nuclear NF-κB activity in the treated aged PPARα−/− mice were comparable to the levels seen in the young PPARα+/+ mice fed the same diet as aged control animals (identical composition without the supplements). Analysis of whole cell extracts of splenocytes from aged and supplemented-aged mice by Western blotting has revealed similar expression of the NF-κB subunit p65 (data not shown). PPARα−/− mice responded to treatment with vitamin E by exhibiting a lowering of nuclear NF-κB levels (Fig. 3A) and a reduction in constitutive IL-6 (Fig. 3B) and IL-12 production (Fig. 3C). The treatment of 15-month-old PPARα−/− mice with DHEAS or WY-14,643, however, had no modifying influence on splenic NF-κB activity (Fig. 3A) or pro-inflammatory cytokine production (Fig. 3, B and C). Reductions in splenic NF-κB activity and cytokine production in PPARα+/+ mice, but not in PPARα−/− mice, were observed following the systemic treatment of aged animals with DHEAS or WY-14,643 for only 3 days, while both PPARα+/+ and PPARα−/− mice responded rapidly to supplementation with additional vitamin E (data not shown). These results strongly suggest that a functional PPARα is required to promote the beneficial effects of DHEAS or WY-14,643 treatment of aged mice. As expected, the capacity of antioxidant supplementation of aged mice with vitamin E to correct the age-associated dysregulation in the
NF-κB system is independent of PPARα influences, as this treatment was effective in both the PPARα+/+ and PPARα−/− mice.

Supershift assays of nuclear extracts obtained from aged PPARα+/+ mice revealed the presence of both p50 and p65 subunits, with an apparent absence of cRel (Fig. 4). The presence of two supershifted bands upon the addition of the α-p50 antibody indicates the presence of both p50/p50 homodimers and, because of its similar gel mobility to that observed upon the addition of α-p65, p50/p65 heterodimers. This conclusion is similar to the observations made by Supakar et al. (53) in their studies of age-associated elevations in liver NF-κB activity and is supported by the finding that the addition of both α-p50 and α-p65 antibodies resulted in the clear appearance of two supershifted bands. Similar results were seen in splenic nuclear extracts obtained from 15-month-old PPARα−/− mice (data not shown). These findings, in addition to the observed elevations in IL-6 and IL-12 production, imply that the NF-κB found in the nuclear extracts of splenocytes from aged mice is indeed capable of transcription-regulating activities.

The Therapeutic Administration of DHEAS or WY-14,643 Requires a Functional PPARα to Lower the Levels of NF-κB Activity and Inflammatory Cytokine Production in Redox-imbalanced Adult Mice—Dietary supplementation with antioxidants, in particular vitamin E, has been well documented to have beneficial effects on numerous physiologic parameters both during aging and in a number of pro-oxidant disease states (54–57). In our hands, dietary supplementation of aged mice with vitamin E was capable of eliciting its antioxidant effects independent of a functional PPARα. We therefore questioned whether a depletion of vitamin E from the diets of 2-month-old PPARα+/+ and PPARα−/− mice might rapidly promote a physiologically aged phenotype in a chronologically young animal. Mice were fed a control diet or a diet deficient in vitamin E for a period of 4 weeks. These diets were identical, except that a tocopherol-stripped fat source and vitamin mix containing no added α-tocopherol were substituted for the normal fat and vitamin mix, respectively. As expected, the PPARα+/+ and PPARα−/− mice fed control diets showed no measurable nuclear NF-κB activity in their spleens. Both the 3-month-old PPARα+/+ and PPARα−/− animals consuming the vitamin E-deficient diet, however, exhibited nuclear translocation of NF-κB in their spleens (Fig. 5). Supershift assay of splenic nuclear extracts obtained from these mice showed the presence of NF-κB composed of p50 and p65 subunits, similar to what was found in the aged mice (data not shown).

Groups of 2-month-old PPARα+/+ and PPARα−/− mice were maintained on vitamin E-deficient diets for a period of 6 weeks. For the last 2 weeks, subgroups of animals from both diet groups were provided with DHEAS, WY-14,643, or vitamin E supplements. Both PPARα+/+ and PPARα−/− vitamin E-depleted mice demonstrated reduced levels of nuclear NF-κB activity in their spleens following vitamin E supplementation (Fig. 6A). Similarly, PPARα+/+ mice provided with DHEAS or WY-14,643 demonstrated elevated levels of nuclear NF-κB activity that closely resembled the levels found in splenocytes from 2-month-old mice fed the control diet (Fig. 6A). The PPARα−/− mice, however, did not show any reduction in splenic nuclear NF-κB levels following dietary supplementation with either DHEAS or WY-14,643 (Fig. 6A).

The transcription-regulating activity of the NF-κB localized to the nucleus in spleens of mice provided with vitamin E-deficient diets was confirmed by ELISA to measure the abnormal constitutive production of the cytokines IL-6 and IL-12. Splenocytes from young PPARα+/+ and PPARα−/− mice cultured overnight without additional stimulation produced only very low levels of IL-6 (Fig. 6B) and IL-12 (Fig. 6C). However, splenocytes from age-matched PPARα+/+ and PPARα−/− mice maintained on the vitamin E-deficient diet for 6 weeks, which induced the activation of NF-κB, demonstrated the spontaneous production of IL-6 and IL-12. This constitutive pro-inflammatory cytokine production could be effectively reduced in PPARα+/+ mice and partially lowered in the PPARα−/− animals following the reintroduction of a supplemental dose of vitamin E into the diets for a period of 2 weeks. A supplementation with DHEAS or WY-14,643 to the vitamin E-deficient diets resulted in a reduction of the spontaneous IL-6 and IL-12 production only in the PPARα+/+ mice. DHEAS or WY-14,643 administration was completely incapable of lowering the spontaneous production of IL-6 and IL-12 in PPARα−/− mice. These results further implicate that the activation of PPARα is capable of having an anti-oxidant effect in mice rendered redox imbalanced either through dietary manipulation or as a normal result of aging.

Activation of PPARα Modulates Tissue Levels of Lipid Peroxides—We and others have previously observed elevated levels of lipid peroxides in the tissues of aged experimental animals and humans (29, 32–34, 58). Vitamin E supplementation has been demonstrated to reduce tissue levels of lipid peroxides (34, 59). In addition, our laboratory has demonstrated that administration of vitamin E or specific activators of PPARα is capable of reducing the age-associated elevations in tissue lipid peroxide levels in the spleen and liver (34). Therefore determined the levels of lipid peroxidation in livers from PPARα+/+ and PPARα−/− mice of various ages. Furthermore, we
questioned whether the capacity of DHEAS and WY-14,643 to modulate the amount of cellular lipid peroxides is through the activation of \( \text{PPAR} \alpha \). The TBARS assay was used to measure tissue lipid peroxide levels in liver homogenates from \( \text{PPAR} \alpha^+/+ \) and \( \text{PPAR} \alpha^-/- \) mice that were young (3 months old), middle aged (12 months old), aged (24 months old), or aged but provided supplementation with DHEAS, WY-14,643, or vitamin E. The lipid peroxide levels in liver homogenates were measured either directly (Fig. 7A) or following in vitro oxidation in the presence of iron (Fig. 7B). At 3 months of age, both \( \text{PPAR} \alpha^+/+ \) and \( \text{PPAR} \alpha^-/- \) mice exhibited similarly low levels of tissue lipid peroxidation. By 12 months of age, however, the \( \text{PPAR} \alpha^-/- \) mice exhibited approximately 2.5-fold elevations in liver TBARS levels than their age-matched \( \text{PPAR} \alpha^+/+ \) counterparts. At 24 months of age, both strains of mice exhibited elevated TBARS levels both under basal conditions or subsequent to in vitro oxidation. Supplementation of aged \( \text{PPAR} \alpha^+/+ \) mice with DHEAS or WY-14,643 was capable of reducing tissue lipid peroxides to levels seen in young animals, while similar treatment of aged \( \text{PPAR} \alpha^-/- \) mice was completely ineffective. As expected, supplementation with the dietary antioxidant vitamin E was capable of reducing the levels of tissue TBARS in both \( \text{PPAR} \alpha^+/+ \) and \( \text{PPAR} \alpha^-/- \) mice, although this treatment appeared to be somewhat more effective in \( \text{PPAR} \alpha^+/+ \) animals. These results suggest that the tissue lipids of \( \text{PPAR} \alpha^-/- \) mice exhibit indications of oxidative damage and are more susceptible to oxidative stress at an earlier age than \( \text{PPAR} \alpha^+/+ \) mice. Supplementation with specific activators of \( \text{PPAR} \alpha \) was only capable of lowering the levels of tissue \( \text{TBARS} \) in both \( \text{PPAR} \alpha^+/+ \) and \( \text{PPAR} \alpha^-/- \) mice, although this treatment appeared to be somewhat more effective in \( \text{PPAR} \alpha^+/+ \) animals. These results suggest that the tissue lipids of \( \text{PPAR} \alpha^-/- \) mice exhibit indications of oxidative damage and are more susceptible to oxidative stress than age-matched \( \text{PPAR} \alpha^+/+ \) mice. 

**Fig. 7.** Age-associated elevations in tissue lipid peroxide levels occur at a younger age in \( \text{PPAR} \alpha^-/- \) mice and are reduced following dietary supplementation with DHEAS or WY-14,643 in aged \( \text{PPAR} \alpha^+/+ \) mice only. Liver homogenates were obtained from \( \text{PPAR} \alpha^+/+ \) (open bars) and \( \text{PPAR} \alpha^-/- \) mice (filled bars) that were 3, 12, or 24 months old, or 24 months old treated with a 2-week regimen of DHEAS, WY-14,643, or vitamin E. TBARS assays were performed either immediately upon sacrifice (A) or following in vitro oxidation in the presence of 100 \( \mu \text{M} \) iron for 60 min at 37 °C (B). Data are the mean values ± S.D. of 3 mice/group. The absence of error bars implies small S.D. values.
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Fig. 8. Transcript levels of PPARα are reduced in aged mice and are elevated following dietary supplementation with DHEAS or vitamin E. To evaluate the levels of PPARα, acyl-CoA oxidase, catalase, and β-actin, RT-PCR was performed on mRNA extracted from the splenocytes from C57BL/6 mice that were 2 or 24 months old, or 24 months old treated with a 2-week regimen of DHEAS (A) or vitamin E (B). β-Actin was employed to ensure equal loading of cDNA. Data are representative of three separate experiments employing two mice per group.

DHEAS Supplementation—Examination of mRNA obtained from the spleens of young, aged, and DHEAS-treated aged C57BL/6 strain mice revealed an age-associated decline in the levels of mRNA encoding PPARα, which was accompanied by a similar decrease in mRNA for the peroxisome-associated enzymes acyl-CoA oxidase and catalase (Fig. 8A). The levels of PPARα mRNA in splenocytes from young mice was determined by densitometric analysis to be between 2.5- and 10-fold higher than in splenocytes from aged animals (data not shown). Therapeutic treatment of aged C57BL/6 mice with DHEAS for 2 weeks, which we have previously demonstrated to reduce the spontaneous activation of NF-κB and NF-κB-driven genes in the spleen (34) resulted in the up-regulation of PPARα, acyl-CoA oxidase, and catalase mRNA to levels normally observed in young mice (Fig. 8A). Furthermore, aged C57BL/6 mice supplemented for 2 weeks with vitamin E also demonstrated elevated expression of mRNA for PPARα (Fig. 8B), suggesting that balancing the cellular redox state may provide a level of transcriptional regulation for this gene. The prooxidant state observed in the cells of aged animals may therefore be a cause of the age-associated reductions in PPARα gene expression. Reciprocally, reductions in PPARα gene expression may, in part, contribute to the prooxidant phenotype of aging through an age-associated deficiency in the efficient modulation of cellular redox state.

DISCUSSION

Elevated levels of cellular oxidative stress contribute to the pathophysiology of a number of clinical conditions, disease states, and aging. In addition to causing damage to cellular constituents, recent evidence suggests that reactive oxygen species can alter cellular function through their ability to affect signal transduction processes (60). Our laboratory has recently demonstrated that aged C57BL/6 strain mice express a markedly elevated activity of the redox-regulated transcription factor NF-κB in a number of lymphoid organs when compared with young adult controls (34). Activities of dysregulated cytokines and proteins under NF-κB control could be responsible for changes in immune competence and may contribute to other diseases that accompany aging (61–66). Supplementation the diets of aged mice with modest doses of chemical agents capable of activating PPARα reduced the nuclear activity of NF-κB to levels seen in young animals. This was paralleled by a correction of the dysregulated constitutive expression of a number of NF-κB-regulated cytokines and other proteins (see Ref. 34, and references therein). The experiments described herein were designed to question whether the in vivo NF-κB-correcting effects resulting from treatment of aged and redox-imbalanced mice with PPARα activators is mediated through mechanisms that require a functional PPARα.

Activation of PPARα in vivo causes an up-regulation of the mRNA and protein levels of a number of peroxisome- and non-peroxisome-associated enzymes, and structural proteins. Included among these induced genes are enzymes responsible for fatty acid β-oxidation, bile acid and glycerolipid synthesis, amino acid metabolism, certain apolipoproteins and fatty acid-binding proteins, lipoprotein lipase, the antioxidant enzymes catalase and Cu,Zn-superoxide dismutase, and mediators of the glutathione pathway (17, 22). While the β-oxidative enzymes induced by PPARα activators are themselves capable of generating hydrogen peroxide, induction of the antioxidant enzyme catalase appears to be able to effectively counterbalance this effect (67, 68). Following administration of agents capable of activating PPARα, the catalase activity within the cytosol of liver and kidney cells was found to increase greater than 5-fold (69). Induces of cellular oxidative stress are not demonstrated to be altered following the administration of modest doses of PPARα activators, indicative of the enzymatic antioxidant capacity of the induced catalase (70).

Activators of PPARα have now been implicated in the protection against pro-oxidant-mediated damage induced by a number of systems. These include protection against transition metal-induced oxidation of LDL-associated lipids, CCl4-mediated hepatotoxicity, and others (71–76). Recent evidence also suggests that PPARα is capable of regulating plasma levels of lipid peroxides (77), indices of oxygen radical-mediated damage, and that administration of the PPARα activator bezafibrate reduces circulating lipid peroxide levels (78). PPARα activation appears to be capable of modulating the duration of an inflammatory response, probably through the transcriptional up-regulation of enzymes necessary for the metabolic clearance of leukotrienes as PPARα−/− mice were found to elicit an extended response to inflammatory stimuli (17).

The cellular activation of PPARγ has been demonstrated to antagonize activities by a number of transcription factors, including NF-κB, AP-1, and STATs (24, 25). These effects were possibly achieved through a sequestration of essential transcription coactivators such as p300, Src-1, or CBP, or via direct protein-protein or protein-DNA interactions involving PPARγ itself. PPARα also forms stable interactions with other transcription factor complexes, thereby exerting an inhibitory influence on cellular signaling processes (13, 26, 27). Recently, it was demonstrated, in human aortic smooth muscle cells cultured in vitro, that specific activators of PPARα are capable of inhibiting IL-1-induced IL-6 and prostaglandin production. PPARα activation was also able to inhibit cyclooxygenase-2 expression, primarily through the repression of NF-κB transcription activation (28). For any single or combination of the aforementioned reasons, providing aged animals with specific activators of PPARα results in reductions in NF-κB activity and, subsequently, causes declines in the expression of NF-κB-regulated genes.

The observed age-associated declines in PPARα expression may occur for a number of reasons. The gene encoding PPARα is under the transcriptional control of glucocorticoids (GCS) (79). As such, PPARα levels fluctuate in rhythm with circulat-
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It remains to be established whether the administration of PPARα-specific activators to mice with an intact PPARα gene elicits its beneficial effects directly within the spleen or in another organ(s) with higher levels of PPARα expression, such as in the liver (3, 5). Either of these primary sites of PPARα-ligand interaction could allow for the re-establishment of an appropriate redox balance in certain individual cells within a particular organ and possibly in the whole animal. The cells which are beneficially affected by PPARα activators are likely to be those expressing PPARα, as well as those cells in the immediate vicinity of PPARα-expressing cells. Recent work by our laboratory and others has provided convincing evidence that consideration of the roles of PPARα, its activators, and PPARα-regulated genes may have important clinical applications toward maintaining redox balance during aging and re-establishing redox balance caused by pro-inflammatory or oxidant stress-related disease states. If similar age-associated changes are observed in humans, it might offer an explanation for the increased incidence of a number of disease states with aging.

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REFERENCES


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