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PPAR-α Expression Inversely Correlates with Inflammatory Cytokines IL-1β and TNF-α in Aging Rats

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Dehydroepiandrosterone (DHEAS) was given the name "fountain of youth" in reference to its beneficial properties in memory, cognition and aging. Cultured cell studies showed that DHEAS may mediate its action by counteracting aging-associated inflammation via PPAR-α activation. In the present study, we demonstrated an age-dependent increase in IL-1β and TNF-α expression in the brain and the spleen of aging rats, while PPAR-α expression was decreased in the spleen of 18 month-old rats. Oral treatment with DHEAS increased PPAR-α mRNA in 3 month-old rats and decreased PPAR-α protein expression in 18 month-old rats in the spleen. In contrast, DHEAS did not alter cytokine expression in spleen and brain of the three age groups. These findings underline a differential role for DHEAS in PPAR-α expression that is age-dependent, and also, that beneficial effects of DHEAS on cognitive function are unlikely mediated by a decrease in cytokine expression.

KEY WORDS: Aging; cytokine; DHEAS; IL-1β; inflammation; PPAR-α; TNF-α.

INTRODUCTION

Several reports reveal that aging leads to an increase in the basal expression of inflammatory cytokines (1–4). In addition, prospective studies have shown that the level of the inflammatory cytokine IL-6 can be correlated with severity of dementia in Down's syndrome, Alzheimer's disease and acquired immune deficiency syndrome. Based on these observations, it appears that high levels of inflammatory mediators can be deleterious for the central nervous system. The natural hormone DHEAS was shown to mediate anti-inflammatory effects in cultured cells (5). For instance, DHEAS treatment was effective at reducing LPS-induced TNF-α production in astrocytes and microglia (6). Furthermore, a report showed that a 2-week treatment with DHEAS reduces IL-6, IL-12 and lipid oxidation in cultured splenocytes isolated from 15-month old mice (5). This effect was dependent on peroxisome proliferator-activated receptor alpha (PPAR-α) expression as splenocytes extracted from PPAR-α deficient mice were unresponsive to DHEAS treatment (5).

PPAR-α belongs to the nuclear steroid hormone receptor superfamily. Three PPAR isotypes have been identified; PPAR-α, PPAR-γ, PPAR-δ, each with a high degree of homology but differing in tissue expression (7). Physiologically, PPAR expression has been found to be important for maintenance of cellular redox balance and lipid metabolism (8). Evidence supporting a role for PPAR-α in inflammation

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came from a report showing that arachidonic acid-induced ear swelling in PPAR-α deficient mice lasted longer than in wild-type mice (9). A number of studies have demonstrated that activation of PPAR-α or PPAR-γ can down-regulate the expression of IL-1β, TNF-α and inducible nitric oxide synthase in *in vitro* and *in vivo* studies (5,10–15).

To the best of our knowledge, DHEAS effect on CNS cytokine expression has not been analyzed in an *in vivo* aging paradigm. Therefore, in the present study we sought to investigate the impact of aging on PPAR-α and cytokine expression in the CNS as well as the effects of DHEAS treatment. We chose to examine the CNS and spleen in young (3-month old), adult (6-month old) and old F344 rats (18-month old). The aging F344 rat strain was chosen since this paradigm recapitulates several aspects of aging in humans (16). The spleen was used as a peripheral immune system control, since splenocytes have been shown to be responsive to DHEAS treatment (5).

EXPERIMENTAL PROCEDURES

Animals and Treatment

Three, 6, and 18 month-old female Fischer 344 rats were obtained from the National Institute of Aging. Rats were housed according to CCAC regulations with a 12 h light dark cycle. Dehydroepiandrosterone 3-sulfate (DHEAS) (Sigma, Oakville, ON) was dissolved in methanol and subsequently diluted in water to a final concentration of 1 mg/ml. DHEAS solution was accessible to rats *ad libitum*. During a 14 day time period, DHEAS intake was approximately 12.5 mg/day. After treatment, DHEAS-treated rats (n = 5–6 per age group) and control rats (n = 4–6 per age group) were anesthetized with intraperitoneal injection of pentobarbital (Somnotol) (1.5 ml/kg) and transcardial perfusion was performed using phosphate-buffered saline (PBS) containing 1% heparin. Tissues were harvested immediately.

Western Blotting

Tissues were homogenized in buffer 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris and 1 mM EDTA. Proteins were separated on a 10% or 14% SDS-PAGE and transblotted onto a nitrocellulose membrane. Membranes were blocked in non-fat milk (5%) for 1 h and incubated overnight in either rabbit polyclonal anti-rat IL-1β (1:500; Biosource International, Camarillo, CA, USA), rat monoclonal anti-mouse TNF-α (1:500; BD Biosciences, Mississauga, ON, Canada), or goat polyclonal anti-PPAR-α antibodies (1:500; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Membranes were washed with PBS/0.075% Tween and incubated with appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnologies). Membranes were washed with PBS/0.075% and detected using enhanced chemiluminescence. Results were quantified by densitometric analysis using the NIH 1.63 quantitative analysis program and normalized to GAPDH band intensity.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Tissues were homogenized in guanidium isothiocyanate (InVitrogen, Burlington, ON, Canada) with subsequent phenol:chloroform extraction. 100% ethanol and 1N acetic acid were used to precipitate total RNA followed by three ethanol washes. The RNA quantity was assessed by measuring the absorption at 260 nm and the purity by the 260/280 nm absorbance ratio. RT-PCR was preformed in accordance with the InVitrogen SuperScript First-Strand Synthesis System (InVitrogen). Briefly, DNAse I amplification enzyme (1.5 units) was added to 5 μg of isolated RNA to abrogate DNA contamination. Random hexamers (50 ng) were used as RNA primers and SuperScript II Reverse Transcriptase (50 units) was added to synthesize complementary DNA strand (cDNA). RNAse H (2 units) was then utilized to remove the RNA template. Subsequent amplification of partial cDNA was performed using gene-specific primers: PPAR-α sense 5′-AAGCCATCTTCACGATGCTG-3′, PPAR-α anti-sense 5′-TCAGAGGTCCCTGAACAGTG-3′ (annealing temperature = 60°C); S12 sense 5′-ACGTCAACACTGCTCTACA-3′, S12 anti-sense 5′-CTTTGCCATAGTCCTTAAC-3′ (annealing temperature = 54°C). The PCR reactions were catalyzed by *Taq* DNA polymerase (5 units) (InVitrogen). PCR conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at respective temperature for 30 s and extension at 72°C for 30 s. Finally, amplified fragments were separated by electrophoresis in a 1% agarose gel stained by ethidium bromide. Bands were quantified by densitometric analyses and normalized to S12 band intensity.

RNAse Protection Assay

The RNAse Protection Assay (RPA) was performed as previously described (4). Briefly, the RIBOQUANT rCK-1 template (BD Biosciences, Mississauga, ON, Canada) set was used to create radioactive anti-sense RNA probes. Sample RNAs were incubated with anti-sense RNA probes overnight. RNAse A was then added to the mixture to digest unprotected RNA, which was followed by a precipitation with 100% ethanol. Hybridized RNA/anti-sense RNAs were separated on an acrylamide gel, which was subsequently dried and exposed to a Kodak BioMax MR film. Results were quantified by densitometric analyses and normalized using GAPDH band intensity.

Statistical Analysis

Values are means ± SE. Statistical significances were determined using the Student's t-test on Primer of Biostatistics 3.0 program. $P < 0.05$ was considered significant.

RESULTS

Aging vs. PPAR-α and Cytokine Expression

Given that DHEAS production decreases with aging, we sought to determine whether age also affects PPAR-α expression. The analyses of 3, 6, and 18-month old spleen homogenates revealed a decrease in PPAR-α protein expression of 50%

($P = 0.03$) between the ages of 3 and 18 months and of 40% ($P = 0.08$) between 3- and 6-month old rats (Fig. 1a). In contrast, aging did not modulate PPAR-α protein expression in the brain (Fig. 1b). These findings suggest that aging affects the expression level of PPAR-α in a tissue-dependent manner.

Several studies suggest that aging correlates with an increase in cytokine expression (1–4). In the spleen, IL-1β and TNF-α protein expression increased between the ages of 3 and 18 months by 70% and 170%, respectively ($P = 0.03$; $P < 0.001$; Fig. 2a). Similar results were obtained in the brain, as IL-1β and TNF-α expression were increased between the ages of 3 and 18 months by 190% and 330%, respectively ($P < 0.001$; $P < 0.001$; Fig. 2b). These data are in agreement with previous studies reporting an age-dependent increase in cytokine expression (1–4). Furthermore, these results suggest a disparity between PPAR-α and cytokine protein expression as a measure of inflammation.

DHEAS Effect on PPAR-α and Cytokine Expression

A previous study has shown that spenocytes extracted from DHEAS-treated mice display high PPAR-α expression and low cytokine production (5). In our study, DHEAS treatment decreased PPAR-α protein expression by 60% in 18-month old rats ($P = 0.03$); no effect was observed in 3- and 6-month old rats in the spleen (Fig. 3a). Interestingly, DHEAS treatment increased PPAR-α mRNA expression by 140% ($P = 0.03$) in 3 month-old rats; yet no differences were observed in 6- and 18 month-old rat spleens after the treatment (Fig. 3c). DHEAS treatment did not modify PPAR-α mRNA (Fig. 3d) and protein expression (data not shown) in the brain of the three age groups. The effect of DHEAS on PPAR-α expression was specific to this isoform since PPAR-γ expression was unchanged (data not shown). Similarly, DHEAS did not affect IL-1β and TNF-α expression at the protein or mRNA level in both the spleen (Fig. 4) and brain (data not shown). These findings suggest that despite anti-inflammatory effects described for DHEAS in cultured cells, modulation of *in vivo* cytokine expression may be more complex.

DISCUSSION

One common feature of aging is the increase in inflammatory mediator levels (17). This increase is thought to increase the likelihood for various disorders including Alzheimer's disease. In our study, we

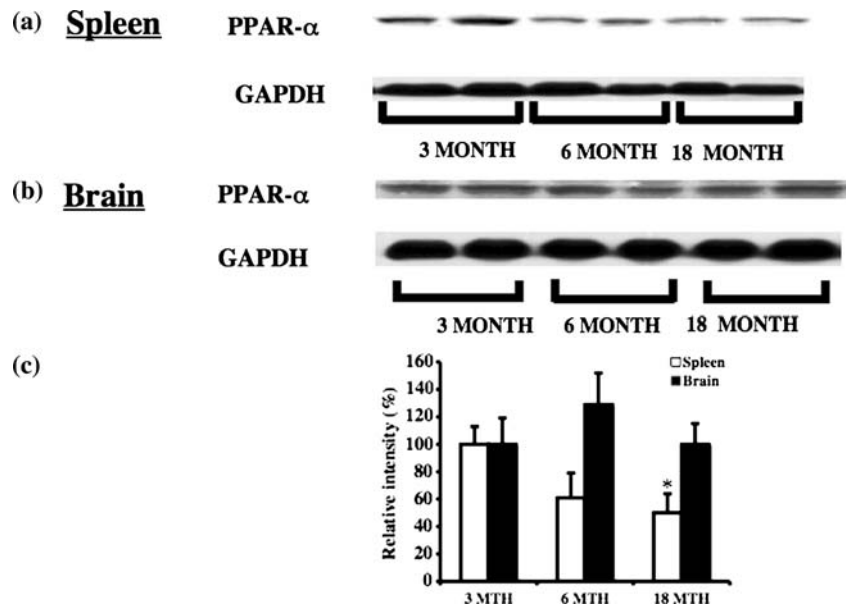

Fig. 1. PPAR-α protein expression in aging rats. (a) In the spleen, Western blot analyses revealed a significant decrease in PPAR-α protein expression between the ages of 3 and 18 months. (b) In the brain, PPAR-α expression did not change with age. GAPDH was utilized to ensure equivalent loading. (c) The graph is representative of Western blot analyses of PPAR-α bands quantified by densitometric analysis using the NIH 1.63 program and normalized to the 3 month-old group. $^*P < 0.05$ as compared to the 3 month-old group.

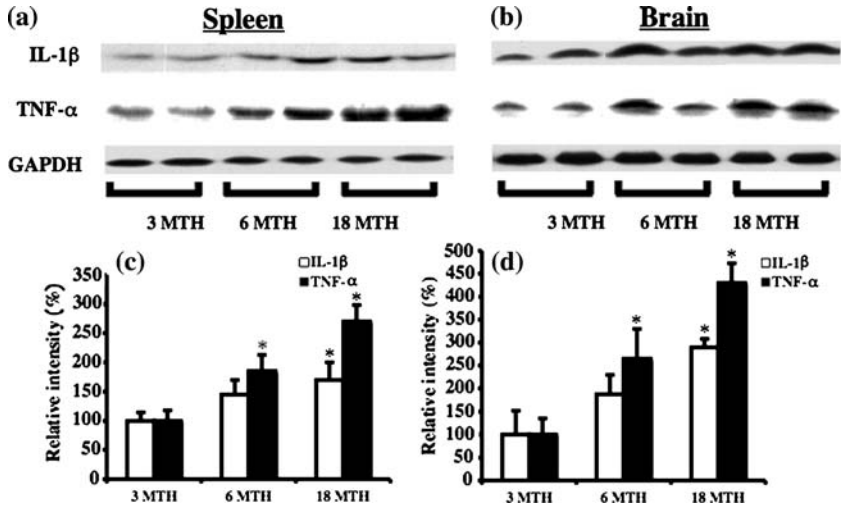

Fig. 2. IL-1β and TNF-α protein expression in aging rats. (a) In the spleen, IL-1β expression in 18-month old rats was significantly higher than that observed in 3-month old rats, while TNF-α was increased in 6- and 18 month-old rats when compared to 3 month-old rats. (b) In the brain, IL-1β and TNF-α expression were higher in 18 month-old rats than that observed in 3 month-old rats. (c, d) Graphs are representative of Western blot analyses of cytokine bands quantified by densitometric analysis using the NIH 1.63 program and normalized to the 3 month-old group. $*P < 0.05$ as compared to the 3 month-old group.

demonstrated an age-dependent increase in IL-1β and TNF-α expression in both the spleen and the brain. Interestingly, this increase was correlated with a decrease in PPAR-α in the spleen. DHEAS treatment was effective at modulating splenic PPAR-α expression as an increase at the mRNA in young rats and a decrease at the protein level in old rats were observed. The literature suggests that some paradigms can

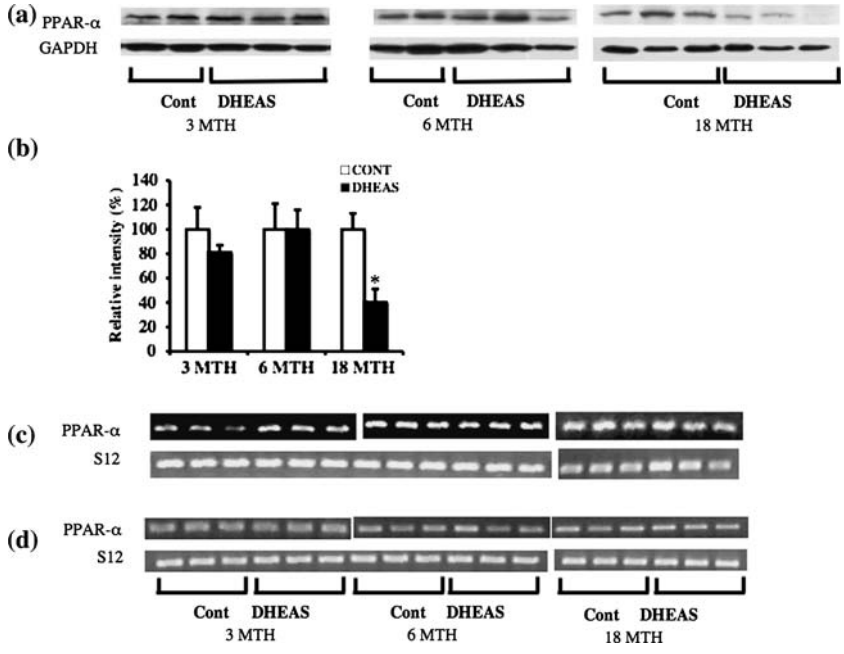

Fig. 3. Effect of DHEAS on PPAR-α protein and mRNA expression in aging rats. Oral treatment with DHEAS for a period of two weeks did not alter PPAR-α protein expression in 3-month and 6-month old rats, while a significant decrease was observed in 18 month-old rats treated with DHEAS compared to the age-matching control group (a). (b) The graph is representative of Western blot analyses of PPAR-α bands quantified by densitometric analysis using the NIH 1.63 program and normalized to the control group for each age. $*P < 0.05$ as compared to the control group. No differences were observed in PPAR-α expression following DHEAS treatment in the brain (data not shown). Using RT-PCR, an increase in PPAR-α mRNA expression in 3 month-old rats was observed after DHEAS treatment in the spleen (c). In the brain, DHEAS did not affect PPAR-α mRNA expression at any age (d). S12 was used as a control for variations in cDNA.

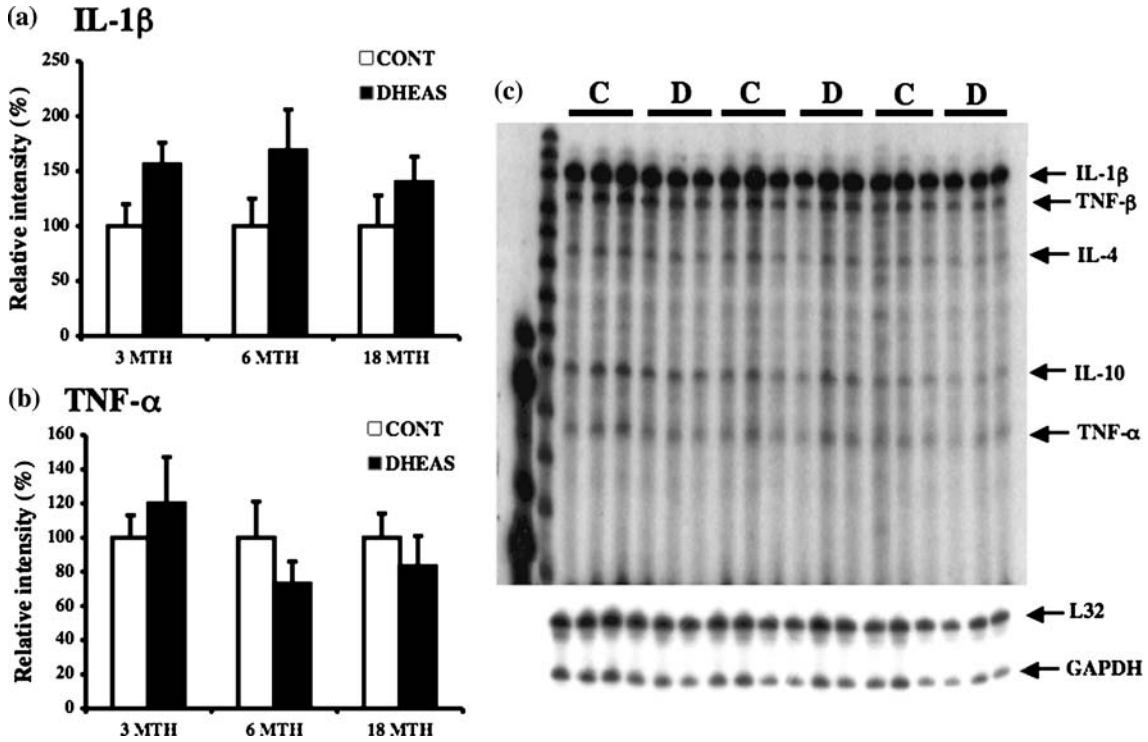

Fig. 4. Effect of DHEAS on cytokine expression in the spleen of aging rats. DHEAS treatment failed to modulate IL-1β (a) and TNF-α (b) protein expression at any age. Graphs are representative of Western blot analyses of cytokine bands quantified by densitometric analysis using the NIH 1.63 program and normalized to the control group. (c) RNAse protection assay showing cytokine mRNA expression in 3-, 6-, and 18-month old rats with or without DHEAS treatment. Anti-sense probes were synthesized using the RIBOQUANT cCK-1 template and hybridized overnight with total RNA followed by digestion with RNAse. Control rat RNA was used to ensure the integrity of the anti-sense probes. No significant differences were observed for the three groups of age (C = control; D = DHEAS). Similar results were obtained for the brain (data not shown).

modulate mRNA without modifying protein expression and *vice versa*. For instance, another isoform of the PPAR family, PPAR-γ, was shown to undergo degradation upon activation with no change detected at the transcriptional level. The degradation was induced by ubiquitination and subsequent processing by the proteasome (18). In contrast, in a murine obesity model, up-regulation of PPAR-γ mRNA does not change the protein level (19). In our model, the differential effect of DHEAS on PPAR-α in aging rats may be due to aging-associated modifications. For instance, aging can be associated with chronic activation of immune cells mediated by the constant activation of NF-kB (20). Furthermore, reports suggest that aging promotes dysfunctionality in the proteolytic pathway (21). In our study, we demonstrated that the protein level of PPAR-α is higher in 3 month-old when compared to 18 month-old rats, which may affect responses to DHEAS treatment.

The effect of DHEAS on splenic PPAR -α expression did not alter inflammatory mediator production as 3, 6 and 18-month old rats did not display variation in IL-1β and TNF-α expression following treatment. In contrast, one report showed that cultured splenocytes extracted from old mice treated with DHEAS for two weeks display lower IL-6 and IL-12 production when compared to non-treated mice (5). Taken together, these results suggest that DHEAS treatment can yield differential results depending on the conditions investigated, i.e. *ex vivo* vs. *in vivo*. Furthermore, our results suggest that cytokine expression is not solely regulated by PPAR-α expression *in vivo*.

We also found that DHEAS treatment had no effect on cytokine and PPAR-α expression in the CNS of aging rats. One possible explanation is that DHEAS did not enter the CNS. This is unlikely given that DHEAS was shown to migrate from the

periphery to the brain within 15 min as measured by radiolabelled DHEAS (^{3}H and ^{35}S) (22). Furthermore, studies have shown that peripheral administration of DHEAS can modulate cognition and biological interactions in the CNS when given in doses similar to those utilized in our study (23–26). For instance, at doses of 10–20 mg/kg, DHEAS significantly attenuated dizocilpine- and scopolanine-induced impairment in the Y-maze test for spatial working memory by modulating sigma type 1 receptor in mice brains (24,25). Moreover, administration of DHEA for a period of 5 days (40 mg/kg), induced the formation of new neurons in the dentate gyrus and hippocampus of adult rats (26). Finally, daily administration of DHEAS (200 mg/day) for 4 weeks markedly increased CSF levels of DHEAS in humans (27).

In light of this study, it can be concluded that changes in the peripheral and central immune system as a result of aging do not correlate with an imbalance in DHEAS expression as oral administration failed to re-establish basal inflammatory mediator levels in old rats. Furthermore our results support the need for drug studies for the treatment of age-related disorders in appropriately aged animals.

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