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Prevention of age-related changes in rat cortex transcription factor activator protein-1 by hypolipidemic drugs

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Abstract

We sought to investigate if, similar to what has been described in other rodent tissues, ageing changes the activity of several transcription factors, namely signal transducer and activator of transcription, nuclear factor-kappa B (NFκB), activated protein-1 (AP-1) and peroxisome proliferator-activated receptor (PPAR), in cortex of Sprague-Dawley rats. We also investigated if the administration of two hypolipidemic drugs, gemfibrozil (GFB) and atorvastatin (ATV), could prevent those changes. To this purpose, we determined the expression and binding activity of these transcription factors in cortex samples from 3-month and 18-month old male and female rats, and in 18-month old rats of both sexes treated for 21 days with a daily dose of 3 mg GFB/kg or 10 mg ATV/kg. Ageing increased rat cortex NFκB binding activity by 35–40%, and decreased by 22–26% the amount of PPARα in rats of both sexes, while cortex AP-1 binding activity and c-Jun content were reduced only in old females (−26 and −50%, respectively). Cortex cyclooxigenase-2 (COX-2) and receptor for activated C-kinase 1 (RACK1) expression was also reduced by old age. Hypolipidemic drugs prevented the age-related decrease of cortex AP-1 in old females and increased AP-1 binding activity and c-Jun protein in cortex from both old male and female rats. GFB increased also by 80% the cortex PPARα content in old males. Our data indicate that 18-month old rats show signs of cortex biochemical deterioration related to the ageing process, and that hypolipidemic drug administration partially prevents the appearance of some of the age-related changes in cortex biochemistry.

Keywords: Old age; Rat cortex; AP-1; c-Jun; Gemfibrozil; Atorvastatin

1. Introduction

Ageing is associated with immunosenescence, decreased hormonal secretion, hepatic metabolism, lean body and bone mass and increased fat accretion. As a consequence, arthritis, dyslipidemia, atherosclerosis, obesity, type II diabetes and neurodegenerative disease incidence increases with age [1]. Although the molecular mechanisms underlying these changes in body physiology are only partly understood, most, if not all, of these pathologies share a common background of increased inflammatory reactions [2–5].

Old rats develop many metabolic alterations common to aged humans. Thus, the incidence of dyslipidemia, obesity associated to leptin resistance and a progressive failure of insulin-mediated glucose metabolism is common to the aged rats and humans [6]. Others and we have shown that in rodents, old age causes a reduction in the expression and activity of peroxisome proliferator-activated receptor (PPARα) in several tissues and cell types [7–9]. As a consequence, metabolic derangement and changes in immune/inflammatory responses develop in senescent animals.

PPARα and PPARγ are nuclear receptors that, besides being involved in the control of fatty acids and glucose metabolism, share potent anti-inflammatory properties [10–12]. They act as anti-inflammatory molecules by repressing the activity of transcription factors, such as

Abbreviations: AP-1, activator protein-1; APRT, adenosyl phosphoribosyl transferase; COX-2, cyclooxygenase-2; IκBα, inhibitor of kappa B alpha; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; RACK1, receptor for activated C-kinase 1; STAT, signal transducer and activator of transcription.

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nuclear factor kappa B (NFκB), signal transducer and activator of transcription (STAT), nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) [13–16], directly involved in the control of the expression of inflammatory molecules. This cross-talk is bi-directional, and in situations with a deficit of PPAR activity, as in the case of cardiac cells and cells of the immune system of senescent animals, the activity of NFκB and other pro-inflammatory transcription factors is increased [9,17–19].

The aim of the present work was to determine if a similar situation, namely a decrease in PPAR expression and an increase in the activity of pro-inflammatory transcription factors, develops in the cortex of senescent Sprague-Dawley rats. Further, currently used hypolipidemic drugs, such as gemfibrozil (GFB) and atorvastatin (ATV), act totally or partially by increasing PPARα activity [20,21] and share common anti-inflammatory properties [14,22,23]. Thus, we also sought to investigate if the administration of these drugs to rats could prevent the age-related changes in the expression and activity of cortex pro-inflammatory transcription factors.

2. Materials and methods

2.1. Animals

Male and female Sprague-Dawley rats, 3- and 18-month old, were purchased from Criffa, and maintained with water and food ad libitum at constant humidity and temperature with a light/dark cycle of 12 h (8:00 am–8:00 pm) for a minimum of 5 days. After this period of acclimatization, rats were weighed and killed by decapitation under ketamine (100 mg/kg, i.p.) anaesthesia between 9 and 10 a.m. In order to reduce the variability in plasma 17β-estradiol concentrations, female rats were killed in the dioestrus period. For assessing the effect of gemfibrozil and atorvastatin, 24 male and 24 female old rats were randomly assigned to control (CT), GFB- and ATV-treated groups. GFB and ATV were incorporated in the diet at a concentration, adjusted to the age-dependent daily food consumption, as to proportionate a daily therapeutic dose of 3 mg GFB/kg and 10 mg ATV/kg to treated animals. Control and treated diets were prepared as described [24], and fed to the animals for 21 days; after this period, animals were sacrificed as described. Before treatment, the animals were maintained in the animal ward for a minimum of 5 days. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995, 21st July, from the Generalitat de Catalunya.

2.2. Sample preparation

Cortex tissue of each rat was immediately frozen in liquid N_2 and stored at −80 °C until used for the extraction of total RNA. Further, a fresh sample of cortex tissue of each rat was immediately used for obtaining nuclear extracts.

2.3. RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx). Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from RNA samples by mixing 1 μg of total RNA, 125 ng of random hexamers (Roche Diagnostics) as primers in the presence of 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies), 20 U RNAsin (Invitrogen Life Technologies) and 0.5 mM of each dNTP (Sigma-Aldrich Inc.) in a total volume of 20 μl. Samples were incubated at 37 °C for 60 min. A 5 μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 50-μl PCR reaction contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 160 μM dNTPs, 2.5 μCi [^{32}P]-dATP (3000 Ci/mmol, Amersham Biosciences), 1 U of Taq polymerase (Invitrogen Life Technologies), 0.5 μg of each primer and 16 mM Tris–HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60 °C). The sequences of the sense and antisense primers used for amplification are shown in Table 1. The *aprt* gene was used as internal control in the PCR reaction to normalize the results for cyclooxygenase-2 (COX-2). For inducible nitric oxide synthase (iNOS) and receptor for activated C-kinase

Table 1
Sequences of the sense and antisense primers used for gene amplification

Primers	Sense and antisense	PCR product (bp)	GenBank accession number
APRT	5′-AGCTTCCCGGACTTCCCCATC-3′ 5′-GACCACTTTCTGCCCCGGTTC-3′	329	L04970
COX-2	5′-TTACTGCTGAAGCCCACCCCA-3′ 5′-GGTGAACCCAGGTCCTCGCTT-3′	429	L20085
iNOS	5′-GCATGGACCAGTATAAGGCAAGCA-3′ 5′-GCTTCTGGTCGATGTCATGAGCAA-3′	198	AJ230462
RACKI	5′-GGTCACTCCCACTTTGTTAG-3′ 5′-AGAAGCGGACACAAGACA-3′	288	U03390

1 (RACK1), the amplification with APRT was performed in separate tubes and in duplicate. PCR was performed in an MJ Research Thermocycler (Ecogen) equipped with a peltier system and temperature probe. After an initial denaturation for 1 min at 94 °C, PCR was carried out for 18 (RACK1), 32 (iNOS) or 23 (COX-2 and APRT) cycles. Each cycle consisted of denaturation at 92 °C for 1 min, primer annealing at 60 °C and primer extension at 72 °C for 1 min and 50 s. A final 5-min extension step at 72 °C was performed. Five microliters of each PCR sample was electrophoresed on a 1-mm thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Agfa X-ray films (Danny Commercial) to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (see Table 1). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating conditions of PCR amplification for all genes studied. Thus, cDNA amplification was performed in comparative and semi-quantitative conditions [25]. Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

2.4. Isolation of nuclear extracts

Nuclear extracts were isolated using the Dignam method [26] with the modifications described by Sonnenberg et al. [27]. Briefly, fresh cortex tissues were weighed and homogenized by a Potter Elvehjem homogenizer in 4 volumes (w/v) of buffer A containing 0.25 M sucrose, 15 mM Tris–HCl (pH 7.9), 15 mM NaCl, 60 mM KCl, 1 mM EGTA, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine and a mixture of protease inhibitors (0.1 mM phenylmethylsolfonyl fluoride, 1.0 mM dithiothreitol, 5 μg/ml aprotinin and 2 μg/ml leupeptin). Homogenates were incubated for 10 min on ice and centrifuged (2000 × *g*, 10 min, 4 °C). Pellets were resuspended in 4 volumes of buffer B (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and protease inhibitors as above), and then centrifuged at 4000 × *g*, 4 °C, for 10 min. Postnuclear supernatants were used for iNOS, RACK1, COX-2 and IκBα protein determination. Pellets were resuspended in 1 volume of buffer C (0.5 M HEPES (pH 7.9), 0.75 mM $MgCl_2$, 0.5 M KCl, 12.5% glycerol and the protease inhibitors). Homogenates were kept for 30 min at 4 °C under continuous rotary shaking, and then centrifuged at 14,000 × *g* for 30 min at 4 °C. Finally, the resulting supernatants were dialyzed overnight at 4 °C with buffer D (10 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 10 mM KCl, 1.0 mM EDTA, 10% glycerol and the protease inhibitors). Nuclear extracts were collected in microfuge tubes and stored in aliquots at −80 °C. The protein concentration of postnuclear supernatants and nuclear extracts was determined by the method of Bradford [28].

2.5. Electrophoretic mobility shift assays (EMSA)

The DNA sequence of the double-stranded oligonucleotides used were as follows: consensus binding site of a PPAR response element, 5′-CAAAACTAGGTCAAAGGTCA-3′; STAT1 consensus oligonucleotide, 5′-CATGTTATGCATATTCCTGTAAGTG-3′ (Santa Cruz Biotechnology); NFκB consensus oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′; AP-1 consensus oligonucleotide, 5′-CGCTTGATGAGTCAGCCGGAA-3′ (Promega). Oligonucleotides were labeled in the following reaction: 3.5 pmol of oligonucleotide (NFκB and AP-1) or 20 ng (PPAR), 2 μl of 5 × kinase buffer, 10 U of T4 polynucleotide kinase (5 U for PPAR) (Invitrogen Life Technologies), and 3 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml, Amersham Biosciences) incubated at 37 °C for 2 h (1 h for PPAR). The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris–HCl (pH 7.4) and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia) according to the manufacturer's instructions. Ten micrograms of crude nuclear proteins was incubated for 10 min on ice in binding buffer (10 mM Tris–HCl (pH 8.0), 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA (pH 8.0), 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA and 50 μg/ml poly(dI-dC)), in a final volume of 15 μl. Labeled probe (approximately 50,000 cpm) was added and the reaction was incubated for 20 min at room temperature. Where indicated, non-radioactive specific competitor oligonucleotide was added before the addition of labeled probe and incubated for 15 min on ice. For supershift assays, 4 μg of antibody was also added before the incubation with labeled probe for a further 90 min at 4 °C. Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). Antibodies against c-Jun and Octamer motif-1 transcription factor (Oct-1) were from Santa Cruz Biotechnology. p65 and p50 antibodies were kindly provided by Dra. Nancy Rice (Uriach Laboratories).

2.6. Western blot analysis

Postnuclear supernatant (25 μg for iNOS, 10 μg for RACK1 and 40 μg for COX-2 and IκBα determinations) or crude nuclear extract (50 μg for c-Jun, p65, PPARα and PPARβ determinations) from cortex, were subjected to SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore) and incubated overnight at 4 °C with 5% non-fat milk solution. Membranes were then incubated with the primary antibody raised against PPARα (dilution 1:1000) (obtained from Dr. Wahli's Laboratory), PPARβ (dilution 1:200) and c-Jun (dilution 1:200) (Santa Cruz Biotechnology), p65 (dilution 1:500), iNOS (dilution 1:1000) and RACK1 (dilution 1:2500) (BD Bioscience, Transduction Laboratories, USA) or against COX-2 (dilution 1:500) (Chemicon International). Detection was

achieved using the ECL chemiluminescence kit for HRP (Biological Industries). To confirm the uniformity of protein loading in each lane, the blots were stained with Pounceau S [29]. Size of detected proteins was estimated using protein molecular mass standards (Life Technologies).

2.7. Statistics

With the exception of Western blots, performed in duplicate with pooled samples from six different animals, results are the mean of n values ± S.D. (see figure legends). Significant differences between sex-paired animals were established by an unpaired t-test, using the computer program GraphPad-InStat (GraphPad Software V2.03). When the number of animals was too small or the variance was not homogeneous, a non-parametric test was performed (Mann–Whitney test). The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Old age differently affects the binding activity of cortex pro-inflammatory transcription factors in rats

The binding of rat cortex nuclear extracts to a STAT1 response element oligonucleotide produced a specific double band (Fig. 1a, see arrow), as its intensity disappeared in the presence of increasing amounts of unlabeled probe. There was no significant difference in STAT1 response element oligonucleotide binding to cortex nuclear proteins of young and old rats in both females and males (Fig. 1b).

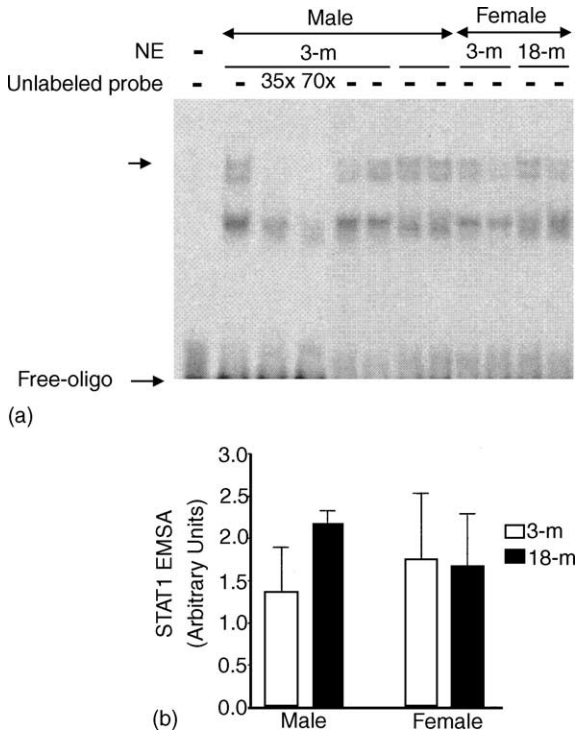

Fig. 1. (a) EMSA assay showing the binding of a STAT1 response element oligonucleotide to rat cortex nuclear extracts (NE) from 3-month (3-m) old male and female and 18-month (18-m) old male and female Sprague-Dawley rats. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from two different animals was loaded. 35× and 70× indicates incubation in the presence of an excess of cold oligonucleotide. (b) Bar-plot of the relative intensity of the specific STAT1 doublet. Each bar represents the mean ± S.D. of four different pooled samples, each one obtained from two different animals.

The binding of rat cortex nuclear extracts to a NFκB response element oligonucleotide produced two specific bands (Fig. 2a), as their intensity disappeared in the presence of increasing amounts of unlabeled probe. Further, p50 and p65 proteins participated in the formation of shifted bands, and they were identified by supershifting with specific antibodies (Fig. 2a). The intensity of the main specific shifted band was increased by 34 and 47% in samples from 18-month old males and females, respectively, versus values obtained in samples from young animals (Fig. 2b). No modification neither in the cortex content of p65 protein, one of the most common component of the NFκB dimer [30] and clearly present in our rat cortex samples (see Fig. 2b), nor in the cortex content of IκBα protein, a physiological inhibitor of NFκB activity [31], was observed in old animals (Fig. 2c and d, respectively).

AP-1 transcription factor gave a clear specific band, that disappeared in the presence of a specific c-Jun antibody, when rat cortex nuclear extracts were incubated with an AP-1 response element oligonucleotide [32] (see Fig. 3a). Old age produced a 26% decrease in AP-1 binding activity, but only in female animals (Fig. 3b). This change was accompanied in the same samples by a 50% reduction in the cortex content of c-Jun protein (Fig. 3c).

3.2. Age-related changes in the cortex expression of inducible iNOS, COX-2 and RACK1 protein

iNOS and COX-2 are two enzymes under transcriptional control by NFκB and AP-1 [33,34], whose products are involved in inflammatory reactions. RACK1 is a protein that binds activated protein kinases C (PKC) and anchors them to specific intracellular sites where target substrates can be phosphorylated, thus controlling PKC-related functions; in brain, RACK1 seems to be involved in synaptogenesis and myelination processes [35]. We determined the effect of ageing in the cortex expression of these proteins.

The amount of COX-2 protein was reduced in the cortex of senescent animals of both sexes by approximately 30% with respect to values presented by sex-matched young animals (Fig. 4a); these changes were accompanied by reductions in the relative mRNA content for COX-2 in 18-month old males (−61%, $P < 0.001$) and females (−40%, ns) (Fig. 4b). While RACK1 protein and mRNA levels were clearly diminished in rat cortex of 18-month old

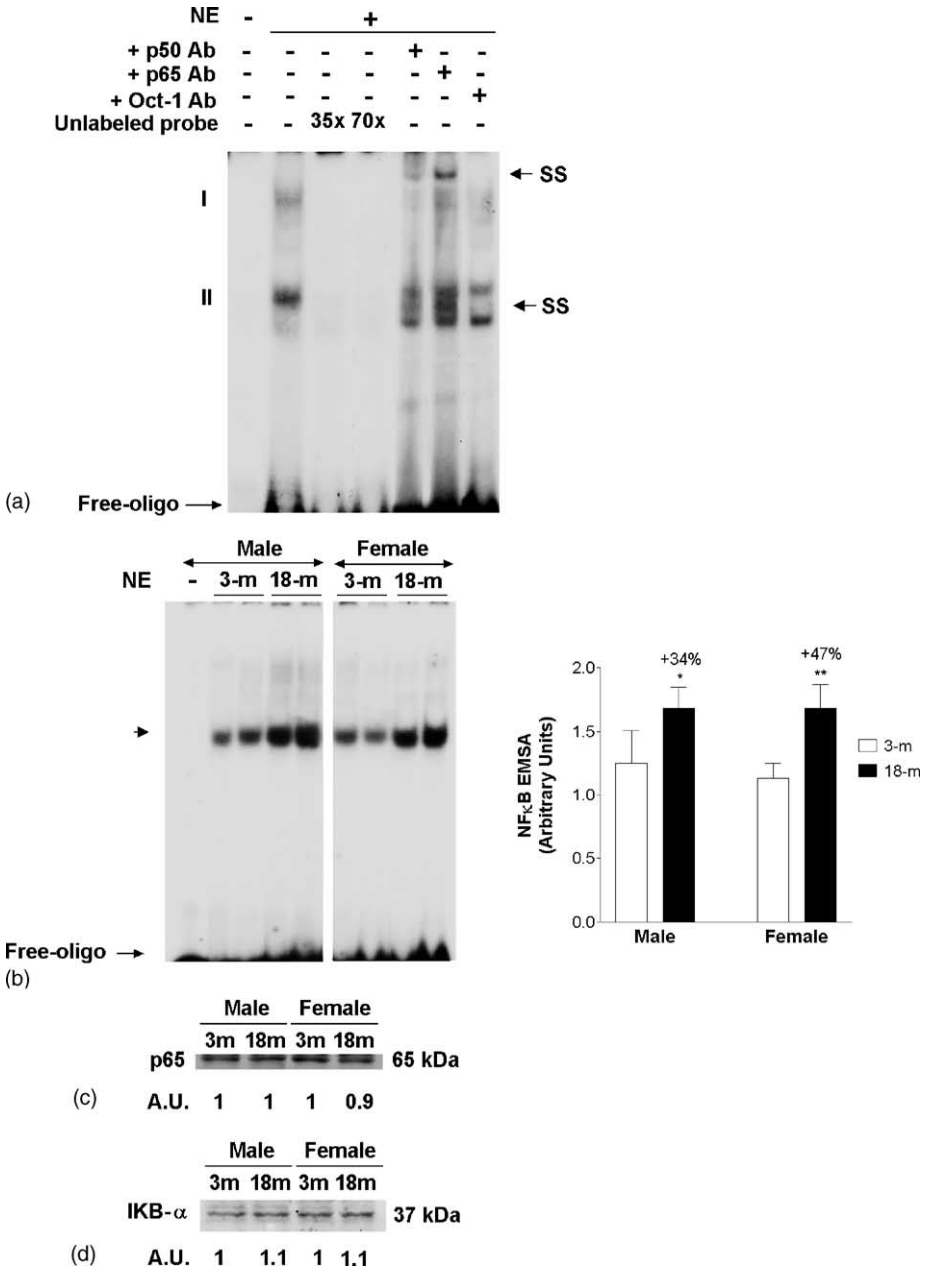

Fig. 2. (a) EMSA assay showing that the binding of rat cortex nuclear extracts to a NFκB response element oligonucleotide produces, at least, two specific bands (I and II), as their intensity disappears in the presence of increasing amounts (35× and 70×) of unlabeled probe. Further, the involvement of proteins p50 and p65 in the formation of specific bands I and II is shown; see the appearance of marked supershifted (SS) bands when cortex nuclear extracts were co-incubated with p50 and p65 antibodies (Ab). Oct-1 Ab was used to demonstrate that supershifts were not due to an unspecific interference produced by the presence in the incubation medium of immunoglobulin proteins. For each line, a pooled sample of cortex nuclear extracts, obtained from two 3-month old male rats was used. (b) In the left side of the figure, an EMSA autoradiography showing the specific NFκB bands corresponding to cortex nuclear extracts from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from two different animals was loaded. In the right part of the figure, a bar-plot of the relative intensity of the specific NFκB band II (see arrow) is shown. Each bar represents the mean ± S.D. of four different pooled samples, each one obtained from two different animals. * $P < 0.05$ and ** $P < 0.01$ vs. sex-matched young controls. (c) and (d) Western blots of p65 and IκBα, respectively, in cortex samples from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U.

males (−30% and −32%, respectively; Fig. 4c and d), a less marked reduction in RACK1 protein was observed in rat cortex of 18-month old females (−20%), with no change in its specific mRNA level (Fig. 4c and d). Regarding iNOS, although we could not detect a measurable signal of its protein in cortex samples of young and old rats (data not shown), ageing increased by 190% the relative levels of iNOS specific mRNA in 18-month old females, while no change was detected in samples from old males (Fig 4e).

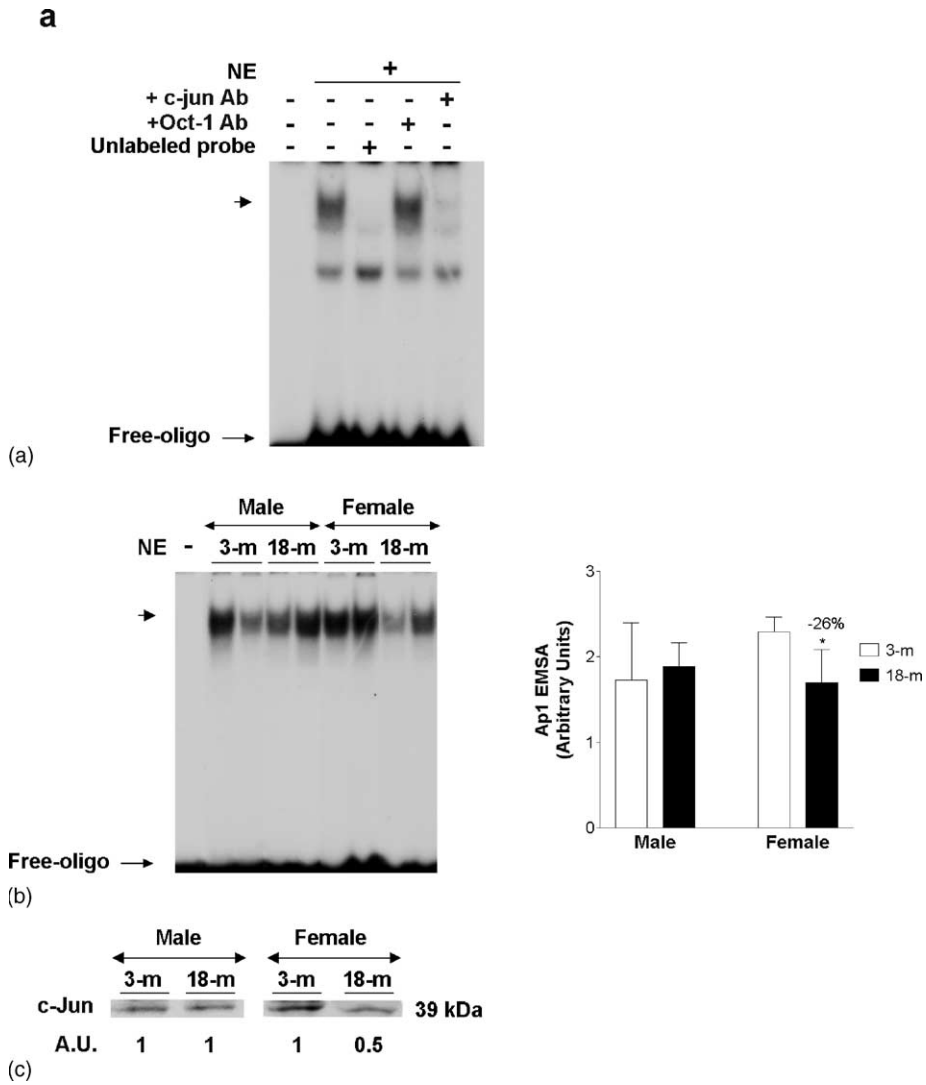

Fig. 3. (a) EMSA assay showing that the binding of rat cortex nuclear extracts to an AP-1 response element oligonucleotide produces a specific band (see arrow), as its intensity disappears in the presence of unlabeled probe. The specific band disappeared when cortex nuclear extracts were co-incubated with a c-Jun antibody, showing the participation of c-Jun protein in its formation. Oct-1 Ab was used to demonstrate that this change was not due to an unspecific interference produced by the presence in the incubation medium of immunoglobulin proteins. For each line, 10 μg of proteins of a pooled sample of cortex nuclear extracts, obtained from two 3-month old male rats was used. (b) In the left side of the figure, an EMSA autoradiography showing the specific AP-1 band (see arrow) corresponding to cortex nuclear extracts from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from two different animals was loaded. In the right part of the figure, a bar-plot of the relative intensity of the specific AP-1 band is shown. Each bar represents the mean ± S.D. of four different pooled samples, each one obtained from two different animals. * $P < 0.05$ vs. sex-matched young controls. (c) Western blot of c-Jun in cortex samples from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U.

3.3. Rat cortex PPARα expression was reduced by old age

Others and we have shown age-related reductions of PPARα in several rodent tissues and cell types [7–9]. Further, we have previously shown that proteins present in nuclear extracts from rat cortex bind to a PPRE oligonucleotide, forming two specific bands in EMSA assays, and that PPARβ participates in the formation of shifted bands [36]. In the present study, although 18-month old rats showed no significant changes in the specific binding of cortex nuclear proteins to a PPRE oligonuleotide (Fig. 5a, bands I and II), old animals of both sexes presented a reduction (−26% in old males and −22% in old females) in the cortex content of PPARα (Fig. 5b). On the contrary, the cortex content of PPARβ, the most abundant PPAR isoform expressed in rat cortex [36–37], was not changed by age (Fig. 5c).

3.4. Treatment with hypolipidemic drugs partially prevented age-related changes in NFκB, AP-1 and PPARα transcription factors

Rat cortex NFκB binding activity, as quantitated by changes in the intensity of the specific band II, was slightly increased in 18-month old rats treated either with atorvas-

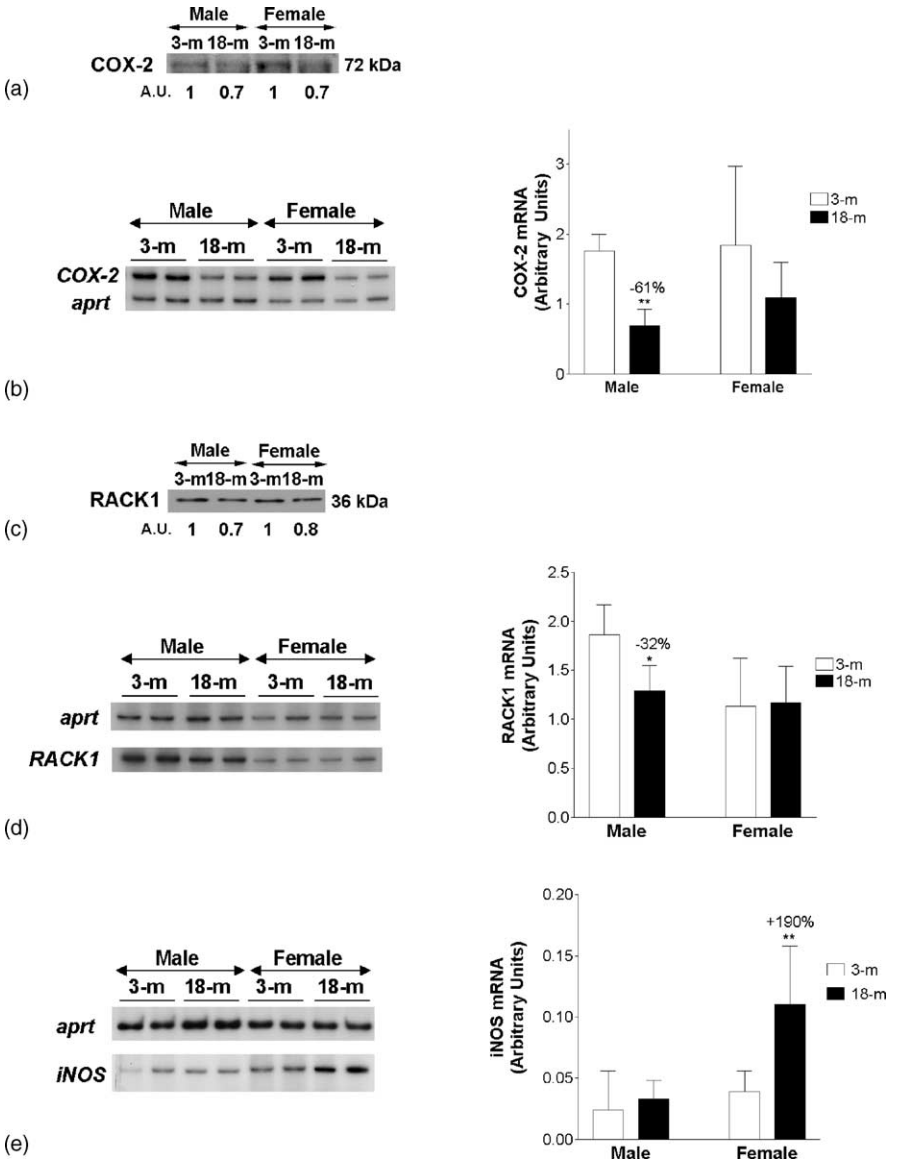

Fig. 4. (a and c) Western blots of COX-2 (a) and RACK1 (c) in cortex samples from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U. (b, d and e) Relative levels of COX-2 (b), RACK1 (d) and iNOS (e) mRNAs in cortex samples from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. Each bar represents the mean ± S.D. of four different samples. A representative autoradiography is presented in the left side of the figure, showing the bands corresponding to each specific gene and that of the *aprt* gene, used as an internal control in the PCR reaction to normalize the results, from cortex samples of two different animals from each age group. * $P < 0.05$ and ** $P < 0.01$ vs. sex-matched young controls.

tatin or gemfibrozil (Fig. 6a, see arrow). Further, rat cortex content of p65 and IκBα proteins was not changed by drug treatment in both sexes (data not shown). Prominent changes were induced by atorvastatin and gemfibrozil on rat cortex AP-1 binding activity; both drugs markedly increased AP-1 band intensity in samples from treated old animals of both sexes (Fig. 6b). Moreover, atorvastatin and gemfibrozil treatment increased the amount of cortex c-Jun protein in samples from old males (100% increase for both drugs) and old females (50 and 80% increase, respectively, for atorvastatin and gemfibrozil) (Fig. 6c). Further, gemfibrozil-treated old animals presented an increase in the cortex content of PPARα protein, especially in the case of gemfibrozil-treated old males, showing a clear 80% increase in PPARα, regarding the values presented by untreated controls (Fig. 6d). Despite these changes, hypolipidemic drug administration to old rats did not modify the expression of cortex iNOS, COX-2 and RACK1 (see Table 2).

4. Discussion

Ageing has been defined as a gradually decreased ability to maintain homeostatic potential and increased risk to die [1]. Eighteen-month old Sprague-Dawley rats present a

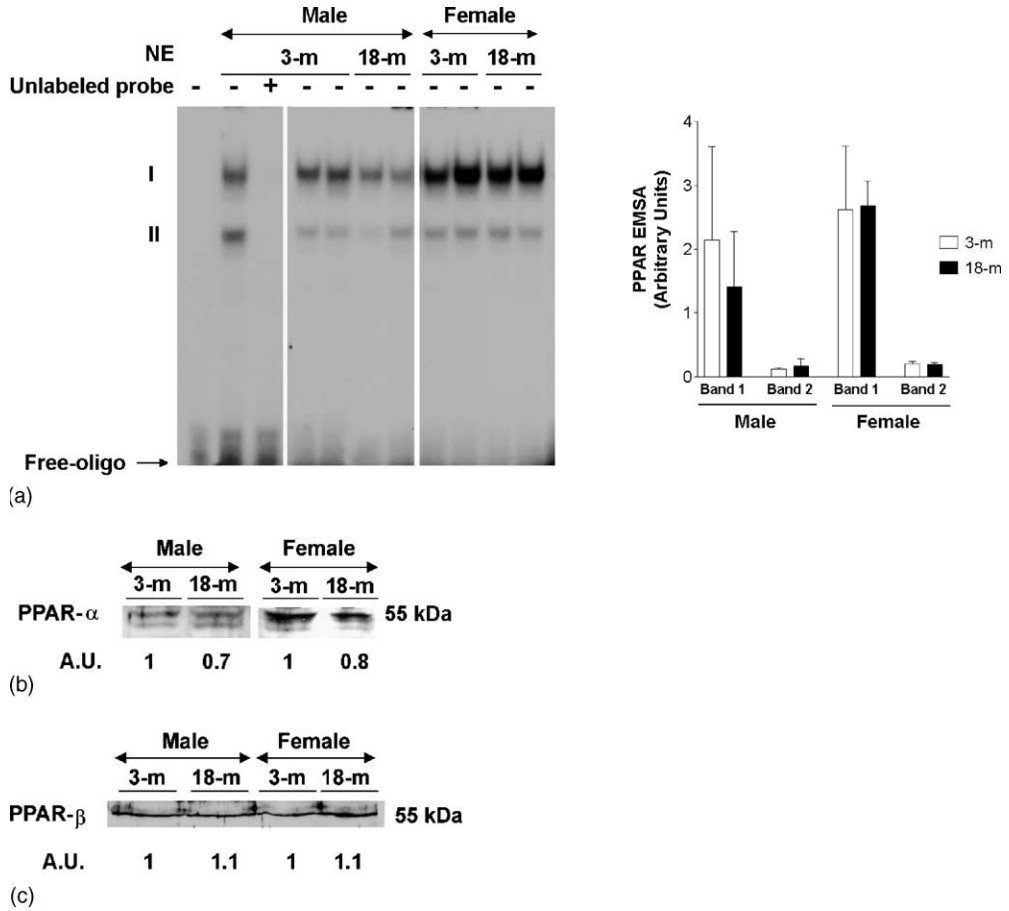

Fig. 5. (a) In the left side of the figure, an EMSA autoradiography showing the two specific PPAR bands (bands I and II), competed in the presence of an excess of unlabeled probe, corresponding to cortex nuclear extracts from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats is presented. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from two different animals was loaded. In the right part of the figure, a bar-plot of the relative intensity of the specific PPAR bands I and II is shown. Each bar represents the mean ± S.D. of four different pooled samples, each one obtained from two different animals. (b and c) Western blot of PPARα (b) and PPARβ (c) in cortex samples from 3-month (3-m) and 18-month (18-m) old male and female Sprague-Dawley rats. A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U.

spontaneous death rate between 10 and 20% (information provided by the supplier), indicating that they are abandoning adulthood and entering the senescence period. We have previously shown that, at this age, the hepatic triglyceride accretion and hypertriglyceridemia presented by male Sprague-Dawley rats are related to a profound reduction in the liver expression and activity of PPARα [7], indicating a marked disarrangement of energy metabolism in these animals. Here we show that 18-month old Sprague-Dawley rats also present changes in the cortex expression and binding activity of several transcription factors related to inflammatory reactions, and that some of these changes are partially prevented by the administration of atorvastatin and gemfibrozil, two well-known hypolipidemic drugs.

Old age introduced in rat cortex a complex pattern of changes in the three pro-inflammatory transcription factors studied, namely STAT1, NFκB and AP-1. STAT1 binding activity remained unchanged, while NFκB binding activity was increased in old male and female rats, and AP-1 binding activity and c-Jun content were decreased only in cortex samples from old females. These changes are not properly attributed to an age-related increase in cortex

Table 2
Effect of atorvastatin (ATV) and gemfibrozil (GFB) administration to 18-month old rats on cortex RACK1, COX-2 protein and iNOS mRNA levels; CT: values corresponding to 18-month untreated rats

	Male			Female		
	CT	ATV	GFB	CT	ATV	GFB
RACK1 protein AU	0.71	0.83	0.64	0.50	0.53	0.44
COX-2 protein AU	1.12	1.21	1.08	0.85	0.89	0.80
iNOS mRNA AU	–	–	–	0.30 ± 0.11	0.26 ± 0.08	0.38 ± 0.06

AU: arbitrary units.

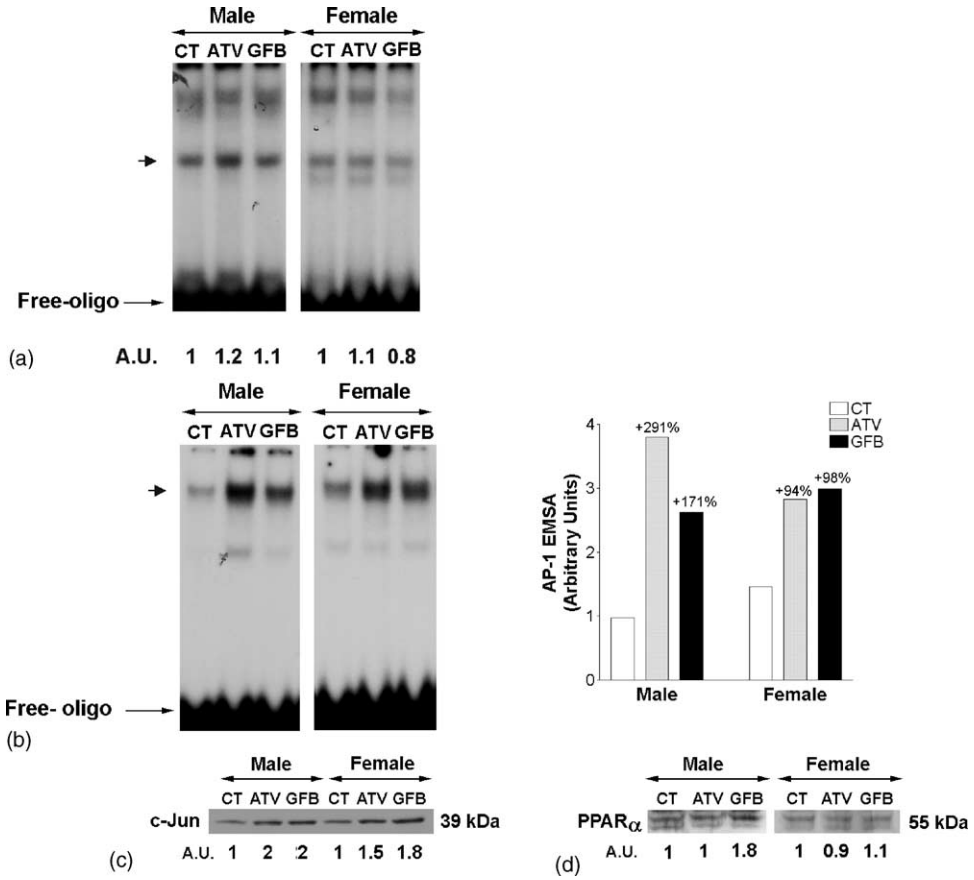

Fig. 6. (a) EMSA autoradiography showing the specific NFκB band II corresponding to cortex nuclear extracts from 18-month old male and female Sprague-Dawley rats control (CT) or treated with atorvastatin (ATV) or gemfibrozil (GFB) is presented. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from six different animals was loaded. A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U. (b) In the left side of the figure, an EMSA autoradiography showing the specific AP-1 band corresponding to cortex nuclear extracts from 18-month old male and female Sprague-Dawley rats control (CT) or treated with atorvastatin (ATV) or gemfibrozil (GFB) is presented. In each line, 10 μg of proteins from a pool of cortex nuclear extracts from six different animals was loaded. In the right part of the figure, a bar-plot of the relative intensity of the AP-1 band I is shown. (c and d) Western blots of c-Jun and PPARα proteins, respectively, in cortex samples from 18-month old male and female Sprague-Dawley rats control (CT) or treated with atorvastatin (ATV) or gemfibrozil (GFB). A.U.: arbitrary units; for each sex, the intensity of the band corresponding to young rats is set to 1 A.U.

inflammatory reactions. Consequently, the cortex expression of two genes, *inos* and *cox-2*, reportedly under transcriptional control by NFκB and AP-1 [33,34], was only increased in old female rats, in the case of *inos*, or even decreased in old rats of both sexes, in the case of *cox-2*. Regarding RACK1, a protein whose expression is not directly related to inflammatory processes [35], old age reduced its cortex expression in male and female rats, in accordance with previous reports [38]. The age-related increase in cortex NFκB binding activity is consistent with similar changes detected in other tissues, such as spleen and other lymphoid organs [18,19], cardiac [39] and endothelial [40] tissues from aged rodents. Although previous reports indicate no age-related changes in AP-1 activity, they have been performed mostly with hippocampus or hypothalamus samples from male rats [41,42]; our results indicate that old age reduces rat AP-1 activity, but only in cortex from female animals. Moreover, the change in AP-1 activity was accompanied by a quantitative reduction, in the same samples, of the cortex amount of c-Jun protein [32].

NFκB has been shown to be activated in multiple types of central nervous system acute lesions, as well as in chronic neurodegenerative conditions [43]; c-Jun and its related AP-1 activity participates actively in the control of neuronal apoptosis, and its expression has been associated with regeneration of axons in the adult peripheral nervous system [32,44]; further, deficiency in RACK1 impairs protein kinase C and the cortex signal transduction systems associated to its activity [38]. Altogether, these data indicate that 18-month old rats show signs of cortex biochemical deterioration directly related to the ageing process.

Gemfibrozil and atorvastatin are drugs widely used for the treatment of dyslipidemia that effectively reduce the incidence of stroke in humans and posses anti-inflammatory properties [22,23,45,46]. Further, experimental data indicate that both drugs penetrate into the central nervous system of rodents at concentrations eliciting pharmacological effects [36,47]. Our present results show that the administration of both drugs to 18-month old male and female rats effectively increases the binding activity of the AP-1 transcription factor in cortex, mainly by increasing

the cortex content of the c-Jun protein, a major constituent of AP-1 [32]. As we have shown that old age causes a reduction in cortex AP-1 binding activity and c-Jun content, at least in female rats, our results indicate that gemfibrozil and atorvastatin administration prevents age-related changes in cortex AP-1 expression and activity.

It has been described that gemfibrozil and atorvastatin reduce NFκB and AP-1 transcriptional activity, probably by a common mechanism mediated by activation of PPARα [14,21,48,49]. Our present work demonstrates a completely opposed effect of gemfibrozil and atorvastatin on AP-1 expression and activity. Although there is a report showing a stimulation of hepatic AP-1 binding activity by a PPARα agonist [50], the changes we have detected in cortex AP-1 of treated old rats are probably not directly mediated through PPARα, given that in our experimental conditions atorvastatin did not modify rat cortex PPARα, and gemfibrozil only clearly increased cortex PPARα in old males, while both old male and female gemfibrozil-treated rats showed a similar increase in AP-1 binding activity and c-Jun content. It has been described that a high-cholesterol content decreases the binding activity of AP-1, but not of NFκB, in rabbit brain [51]; further, there is an age-dependent increase in rat brain 3-hydroxy-3-methylglutaryl-CoA reductase activitity [52]. Thus, although at the present time we cannot confirm this hypothesis, it could be possible that the increase in cortex AP-1 binding and c-Jun expression detected in treated old rats could be secondary to a reduction in cortex cholesterol content induced by the administration of gemfibrozil and atorvastatin.

c-Jun proteins are important regulators of the cell death program in the mammalian nervous system and their biological effects depend on the neuronal type and stage of development [32]. Although, c-Jun may be pro-apoptotic in some neuronal cell types, its increased expression is associated with axonal regeneration in adult peripheral neurons previously axotomised [44]. In this sense, the increased expression of cortex c-Jun protein in old rats after gemfibrozil or atorvastatin administration could be a reflection of an increased neuronal regeneration produced by hypolipidemic drugs.

Besides the increase in AP-1 binding activity and c-Jun content in cortex of old rats, drug-induced changes in other transcription factors, such as NFκB and PPARα were of lesser magnitude or inconsistently presented by treated animals. Only a clear increase in cortex PPARα content produced by gemfibrozil administration to old male rats was detected, probably related to the described increase in PPARα half-life produced by direct agonist interaction [53].

In conclusion, our present work demonstrates that the old age introduce changes in rat cortex NFκB and AP-1 transcription factors and in the expression of cortex iNOS, COX-2 and RACK1. Further, the administration of gemfibrozil and atorvastatin to the aged rats prevented AP-1 changes in old females, and increased cortex AP-1 binding activity and c-Jun content in both old male and female rats, in this way preventing the appearance of some of the age-related changes in cortex biochemistry.

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