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Age-associated increases in oxidative stress and nuclear transcription factor κB activation are attenuated in rat liver by regular exercise

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ABSTRACT

The combined effects of aging and regular physical exercise was investigated on the production of reactive oxygen species (ROS), lipid peroxidation, glutathione status, and the activity of nuclear factor-κB (NF-κB) in rat liver. A group of 24 male F344 rats was divided into the following categories: adult control (18 months), adult exercised (18 months), and aged control (28 months) and aged exercised (28 months). The ROS formation increased as a function of age and exercise training decreased the rate of ROS formation in the two age groups. Significant positive correlation was found between ROS production and lipid peroxidation (LIPOX). The reduced glutathione (GSH) level was higher and the oxidized glutathione (GSSG) level lower in exercised groups compared with the sedentary controls ($P<0.05$). An age-associated increase in NF-κB activity was attenuated by the regular exercise. The content of p50 and p65 subunits of NF-κB increased with age and decreased with exercise training. The content of inhibitory factor-κB was inversely related to NF-κB activation. Regular exercise-induced adaptive responses, including attenuation of an increase in ROS production, LIPOX level, NF-κB activation, and reduced GSH/GSSG ratio, appear to be capable, even in old age, of reducing increases in inflammatory and other detrimental consequences that are often associated with advancing age.

Key words: aging • redox signaling • NF-κB • adaptation • oxidative stress • free radicals

Reactive oxygen species (ROS) are necessary for certain normal biological processes and, at the same time, inevitable potentially harmful products of aerobic metabolism. It has been proven that ROS play an important role in signaling pathways and also regulate transactivation of transcription factors. Hence, ROS appear to be responsible for certain gene expression and cellular responses to internal and external challenges. Nuclear factor κB (NF-κB)

is a redox-sensitive and oxidant-activated transcription factor, which regulates inflammation-related gene expression, viral replication, cell–cell interactions, apoptosis, and proliferation (1–4). The activation pathway of NF-κB often utilizes cytokines as an activator. This leads to the translocation of NF-κB to the nucleus from the cytoplasm upon dissociation from inhibitory molecule I-κB, which is phosporylated by I-κB kinases. The phosphorylated I-κB undergoes ubiquitination and then degradation by the proteasome complex (5). Blocking the I-κB kinase activity can prevent the NF-κB dependent gene expression (6). It should be mentioned, however, that in some cases the activation of NF-κB may be achieved by I-κB degradation without phosphorylation such as in the case of ultraviolet C irradiation (7) or even without I-κB degradation as in the case of oxidative stress in human vascular endothelial cells (8).

According to the free radical theory of aging (9) and its modern version, the oxidative stress theory of aging, there is a disruption in the delicate balance between ROS generation and antioxidant/repair systems with age, which leads to a shift to an oxidative cellular milieu. Hence, aging is associated with an increase in oxidative damage to biomolecules (10–14) and enhanced levels of inflammation (4, 15–16). Available data have shown that aging is accompanied by increased activity of NF-κB in the liver, heart, and kidney, suggesting the involvement of this transcription factor in the age-associated increase in inflammation (17–19).

Regular physical exercise is a natural means to extend healthy life-span and decrease the incidence of a number of diseases (12, 20). Physical exercise can, depending on the intensity and duration, increase the generation of ROS and the activity of antioxidant/repair systems (21, 22), thus altering the cellular redox milieu. The cytokine formation might also be significantly increased by exercise, as regular exercise has been shown to improve the efficiency of the immune system and decrease the probability of inflammation (23).

In the present investigation, we tested the hypothesis that age-associated increases in ROS production and NF-κB activation are attenuated by regular exercise in older animals.

MATERIALS AND METHODS

Materials

All chemical reagents were obtained from Sigma (St. Louis, MO), except where noted. The dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes, Inc. (Eugene, OR). Radionucleotide [γ-^{32}P]-ATP (250 Ci) and Western blotting detection reagents were obtained from Amersham (Piscataway, NJ). Antibodies against p50, p65, and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation (Milford, MA). The Bioxytech LPO-586 kit used for the lipid peroxidation assay was obtained from Oxis International (Portland, OR).

Animals

This experiment followed the guidelines published by the Council of the Physiological Society of Japan. Adult (18 month) and old (28 month) specific pathogen-free male Fischer 344 rats were housed in a climate-controlled laboratory animal facility at the Experimental Animal Center, Faculty of Pharmaceutical Sciences, Toho University (23±1C, 50±5% relative humidity, and

12:12 h light–dark period) and were fed standard rat chow and water ad libitum. During the period of exercise training, described below, the animals were kept in a facility of similar conditions as described above at School of Sports Sciences, Juntendo University.

Exercise training protocol

All animals were familiarized with walking on a motor-driven treadmill (5%, 6–8 m/min, 10 min/day) for 5 days. At the end of this period, animals from each age group were weight-matched and randomly assigned to either a sedentary control or an endurance exercise-trained group. Four experimental groups were formed: 1) adult sedentary control (AC); 2) adult exercise trained (AT); 3) old sedentary control (OC); and 4) old exercise trained (OT). The treadmill training program was intended to exercise both adult and old animals at the same relative exercise intensity (i.e., $\%VO_{2max}$) for the two age groups during the 8-week training period (see Table 1 in 22). Electric shock was rarely used to motivate the animals to run. Both adult and old sedentary control animals were also run once a week for 10 min on a 15% grade at 8 m/min for adult and 6 m/min for old to familiarize them with handling and treadmill running.

Measurement of maximal oxygen consumption

At the end of the seventh week of training, maximal oxygen consumption (VO_{2max}) was measured on all animals with the use of a flow-through open-circuit system. Briefly, individual animals were placed in a sealed treadmill chamber that allowed a unidirectional flow of gas and the progressive exercise test began on a 15% grade at the initial speed used for training (adult, 10 m/min; old, 7.5 m/min). The treadmill speed was increased 5 m/min for adult and 2.5 m/min for old every 3 min until the animal was unable to maintain the required running speed within 21 min. VO_{2max} was defined as the highest VO_2 obtained during the exercise test. Ambient air was pumped through the chamber at a flow rate of 5.5 L/min. Gas was sampled (500 ml/min) from a small mixing chamber located at the back of the treadmill and analyzed for CO_2 and O_2 concentrations via electronic gas analyzers (Minato MG-360, Tokyo, Japan). The gas analyzers were calibrated immediately before and after each test by using standardized gases. Seventy-two hours after the VO_{2max} measurement, animals were anesthetized with pentobarbital sodium (25 mg/kg). The liver was quickly removed, weighed, and frozen with liquid nitrogen. Samples were then stored at –80 C until analysis.

Preparation of homogenate

One gram of liver was homogenized with 5 ml of homogenate buffer containing 20 mM glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 M pepstatin, 80 mg/l trypsin inhibitor, and 100 mM Tris-Cl, pH 7.4, and centrifuged at 900 g at 4°C for 15 min. The supernatants were recentrifuged at 12,000 g at 4°C for 15 min to yield sedimented mitochondrial fraction and postmitochondrial supernatant fraction.

Gel shift assay of NF-κB

DNA binding activity of NF-κB was measured by electrophoretic mobility shift assay (EMSA) as described by Kim et al. (24). Nuclear extracts were prepared as described previously (25). The

oligonucleotide with the sequence of 5′-GAGAGGCAAGGGATTCCCTTAGTTAGGA-3′ was terminally labeled with ^{32}P using [$\gamma^{32}P$]-ATP and T4 polynucleotide kinase. For NF-κB binding assay, 10 μg of nuclear proteins were mixed with the labeled probe in a buffer containing 1.0% Nonidet P40, 5% glycerol, 1 mM MgCl2, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, and 10 mM Tris/HCl, pH 7.5 (Kim et al. 2000). Unspecific binding was blocked by 1 μg of poly(dI-dC)/poly(dI-dC). The mixtures were incubated at room temperature for 20 min, and the [^{32}P]-labeled oligonucleotide-protein complex was separated from the free oligonucleotide by electrophoresis through a 5% native gel in a running buffer containing 50 mM Tris-HCl (pH 8.0), 45 mM sodium borate, and 0.5 mM EDTA. After separation, the gel was vacuum-dried for autoradiography and exposed to Fuji X-ray film for 1 to 2 days at –80 C. To determine the specificity of the nuclear protein binding, competition with the corresponding unlabeled NF-κB oligonucleotide was performed under the same conditions.

Western blot

Western blots were used to measure the contents of NF-κB p50, p65 subunits, and I-κB. Briefly, samples were boiled with gel-loading buffer (0.125 M Tris-Cl; 4% SDS; 10% 2-mercaptoethanol, pH 6.8; 0.2% bromophenol blue) in the ratio 1:1. Total protein equivalents (100 μg) for each sample were separated by SDS-PAGE by using 10% acrylamide gels and were transferred to PVDF membrane in a semi-dry transfer system. The membrane was immediately placed into blocking buffer containing 1% nonfat milk in 10 mM Tris, pH 7.5; 100 mM NaCl; and 0.1% Tween 20. The blot was allowed to block at room temperature for 1 h. The membrane was incubated with rabbit polyclonal anti-IκBα (1:500) for 1 h at 25°C, followed by incubation in an antirabbit IgG-horseradish peroxidase conjugated antibody (1:5000). Western blotting with goat polyclonal anti-p50 (1:500), or goat polyclonal anti-p65 (1:500) was also performed. The secondary antibodies were detected by using enhanced chemiluminescence per the manufacturer's instructions. Pre-stained protein markers were used for molecular weight determinations.

Assessment of oxidative status

The overall ROS generation was determined by using modifications of the dichlorodihydrofluorescein diacetate (H_2DCFDA) staining method (24). The H_2DCFDA was replaced by carboxy-H_2DCFDA. We used oxidation-sensitive carboxy H_2DCFDA and oxidation-insensitive carboxy-DCFDA fluorescent dyes. The oxidation-insensitive dye was used as a control to ensure that changes in the fluorescence seen with the oxidation-sensitive dye were due to changes in ROS production. In brief, oxidation-insensitive and oxidation-sensitive dyes were dissolved at a concentration of 12.5 mM and kept at –80 C in the dark. The solution was freshly diluted with homogenization buffer to 125 μM before use. Diluted dyes were added to liver homogenate (100 μg) in a 96-well plate to achieve a final concentration of 25 μM. The change in fluorescence intensity was monitored at two time points (0 and 30 min) by using a microplate fluorescence reader (Bio-Tek Instruments), at excitation 485 nm/emission 530 nm, as described earlier (19, 24).

GSH and GSSG determination

To assay for GSH, a fluorometric method was used. One mM EDTA-50mM phosphate buffer was added to the supernatant, follwed by o-phthadehyde (24). Afer 20 min at room temperature, the fluorescence was measured at an excitation wavelength of 360 nm and emission wavelength of 460 nm. GSSG was assayed after preincubated with N-ethylmaleimide for 20 min and 0.1 M NaOH was substituted for phosphate buffer.

Lipid peroxidation

Lipid peroxidation was determined by using the Bioxytech LPO586 kit (19). The assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE). To measure MDA plus 4-HNE, samples were incubated with R1 and methanesulfonic acid at 45°C for 45 min and then were centrifuged at 12,000 g for 15 min. After the supernatant was transferred to a 96-well microplate, the optical density was measured at 600 nm by using a microplate reader. The amount of 4-HNE was obtained by the subtraction of the MDA values from the total MDA plus the 4-HNE values.

Statistical analysis

Statistical significance was assessed by using ANOVA, followed by Scheffe's posthoc test. The method of least squares was applied to determine the linear correlation coefficients. The significance level was set at $P < 0.05$.

RESULTS

Body weights, VO_{2max}, and training intensity

The body weights of animals in the exercised groups were significantly lighter than those in the non-exercised groups, 89% and 87% in the adult and aged groups, respectively. Endurance-training resulted in a 39% ($P<0.05$) increase in VO_{2max} in both adult and old animals. The training intensity was estimated to be ~75%VO_{2max} for both adult and old animals as expressed by percent of VO_{2max} that was calculated from the results of the graded exercise test and the relative training intensity.

The regular exercise applied in the present study resulted in increased maximal oxygen uptake in both adult and aged exercised groups compared with non-exercising age-matched groups.

Redox status of the liver

To investigate the age-related redox status, total ROS were measured with DCFDA probe in liver homogenates. Fig. 1*A* depicts a significant increase of ROS with age, which showed a fluorescence intensity increase of ~28% compared with younger rats. To the contrary, exercised adult and old rats, compared with the non exercised same age group, decreased 10 and 9%, respectively. It should be noted the data in Fig. 1*A* show no discernable age-related GSH changes. The plausible explanation is that hepatic GSH levels are a response to various hepatic functions, including detoxificating process.

A marker of aging, lipid peroxidation, is well known as an accompaniment to oxidative damage. It was measured by detecting the amounts of MDA and 4-HNE. As shown in Fig. 1*B*, the levels of MDA and 4-HNE were revealed to demonstrate an increase (16%) with age in the non exercised group, although both adult and old animals in the exercised group had lower levels.

Exercise-dependent decreases in the ROS levels were confirmed by other oxidative markers. Because increases in GSSG, at the expense of GSH, are associated with oxidative stress, the changes of GSH and GSSG levels were assessed. As demonstrated in Fig. 2*A*, old rats showed almost the same GSH level as adult rats. However, exercised rats preserved higher GSH levels when compared with their non-exercised counterparts. Fig. 2*B* shows, as expected, that the GSSG levels are decreased in accordance with increased GSH levels. The overall oxidative status, judged by ROS production, LIPOX level, and GSH/GSSG ratio suggests that the cellular milieu is shifted to a state of higher oxidative stress with age. Regular exercise, on the other hand, can prevent and/or reverse this even in old age.

Changes in redox sensitive NF-κB

The exercise-related activation of NF-κB, EMSA was performed with nuclear protein. Fig. 3 illustrates age-associated increases in the nuclear binding activity of NF-κB in the old group, and the increase is attenuated by exercise. The specificity of the DNA binding of NF-κB complex was studied by using a 100-fold excess of an unlabeled oligonucleotide, which competed with the labeled probe. The data conclusively demonstrate that aging accelerates NF-κB activation, although exercise attenuates age-related alteration of NF-κB.

Cytosolic I-κBα is known to play a major role in the translocation of NF-κB into the nucleus by release of I-κB subunit from NF-κB-I-κB complex, and the I-κB is phosphorylated before it is degraded. As ascertained in the present study (in Fig. 4*A* and *B*), the I-κBα level in the cytosol decreased with age in non-exercised groups, while the level in adult and old exercised groups remained higher than those in the non-exercised groups. Thus, the results indicate that attenuation of NF-κB activation by exercise is interrelated with the change in the amount of I-κBα.

To substantially confirm that the decrease in I-κB protein is associated with the increased nuclear translocation of NF-κB, we examined the nuclear protein level of p50 and p65 components of NF-κB, by immunoblot using corresponding antibodies. Results shown in Fig. 5 clearly demonstrate that the nuclear p50 and p65 were modulated by aging and exercise, verifying that the nuclear translocation of NF-κB is increased with aging and that the exercise prevented this change. Thus, it was corroborated that exercise-related prevention of translocation of NF-κB, that is, p50 and p65, was caused by inhibition of degradation of I-κBα.

DISCUSSION

The present study confirms that aging is associated with a shift in redox state toward an oxidized milieu and clearly demonstrates that regular exercise attenuates the age-related increase in cellular ROS concentration. It appears that aging develops an increased level of ROS, which does not necessary mean a loss of homeostasis, but rather a chronic shift in homeostasis (26).

The balance between ROS generation and elimination occurs at a higher level of ROS production, which could be associated with an increased level of oxidative damage of macromolecules. Indeed, in the current study increased levels of LIPOX were found in the liver and the oxidative DNA damage was elevated in the skeletal muscle of the aged group (22). The chronic shift in cellular redox state might mean significantly reduced ability to cope with oxidative stress, due to the decreased mobility of a buffering system, which naturally involves the thiol system. GSH, γ-glutamyl-cysteinyl-glycine, is one of the main thiol/antioxidant sources of the cell, which is continuously synthesized by the γ-glutamyl cycle. Exercise training increased the level of GSH and the GSG/GSSG ratio, suggesting an increased resistance to oxidative stress. Aging did not significantly affect the GSH/GSSG ratio. The increase in age-associated ROS production could be due to the increased leakage of electrons from the mitochondrial respiratory chain (27), as it has been shown that regular exercise has the capability to decrease the mitochondrial membrane potential. This may account for highly efficient ATP generation and ROS production (28).

Alternative pathways could also explain the beneficial effects of exercise training on ROS production. It has been postulated that the semiquinone is a major autoxidizable electron transport chain component yielding the superoxide radical (29). An age-associated decrease in ubiquinone content occurs, thus possibly reducing the antioxidant activity of cells, which is consistent with the free radical theory of aging (9). Ubiquinone is present mainly in reduced form (30), hence, a quinone reductase, DT-diaphorase, of which activity is up-regulated by regular exercise, (31, 32) might play a role to decrease the rate of ROS production from the mitochondrial electron transport chain.

The significant production of mitochondrial ROS as a result of physical exercise was demonstrated by Davies et al. (33), who described a threefold increase in mitochondrial ROS production after a single bout of exercise. Our data on the decreased generation of ROS after exercise training is not in opposition to this result after a single bout of exercise. On the other hand, an increased level of ROS production, which is observed after a single bout of exercise, is controlled and significantly attenuated as a result of exercise training-induced adaptation (21). An important novel finding of this study is that the rate of ROS generation is down-regulated in the liver, which is a non-exercising tissue, as a result of regular training. It has been demonstrated that regular exercise could significantly enhance the level of antioxidant system and efficiency, hence resulting in decreased level of the accumulation of oxidative damage in various organs (reviewed by 21, 34). Moreover, the exercise-induced induction of heat shock proteins is an important part of the defense system against ROS (35, 36). Therefore, regular exercise offsets the oxidative stress, probably by the combined effects of different systems including the down-regulation of mitochondrial ROS release, increased antioxidant/repair mechanisms, induction of heat shock proteins and via redox regulation.

The present study reveals that the DNA binding activity of NF-κB, which is a key transcription factor, is increasing with age and that regular physical exercise attenuates this increase in the liver. The age-associated increase in NF-κB activity in the liver is in accordance with earlier findings; moreover NF-κB activity is also increased in other tissues including the brain, kidney, gastric mucosa, smooth muscle, and cardiac muscle (17–19, 37, 38). Therefore, NF-κB is a potential factor responsible for the age-associated increase in inflammation (3, 39–41). Hence,

our finding that regular exercise can reduce the age-related increase in NF-κB activation explains the molecular basis for beneficial effects of exercise on the reduction of the incidence of age-associated inflammatory diseases (42).

In the present experimental conditions, an inverse relationship was found between the I-κB protein content and NF-κB activity, suggesting an effective degradation of I-κB after the dissociation of NF-κB.

Taken together, the observations of the present study suggest that regular exercise, even at older ages, results in a significant increase in oxygen uptake and therefore potentially generates more oxidative stress and attenuates, contrary to expectation, the increase in NF-κB activity in the liver of rats. Regular exercise not only prevents the age-associated increase in NF-κB activity, but the down-regulation of ROS production. The associated higher reserve in antioxidant capacity means greater resistance against oxidative-stress related diseases. This might provide another mechanism to demonstrate beneficial effects of regular exercise for older individuals.

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Fig. 1

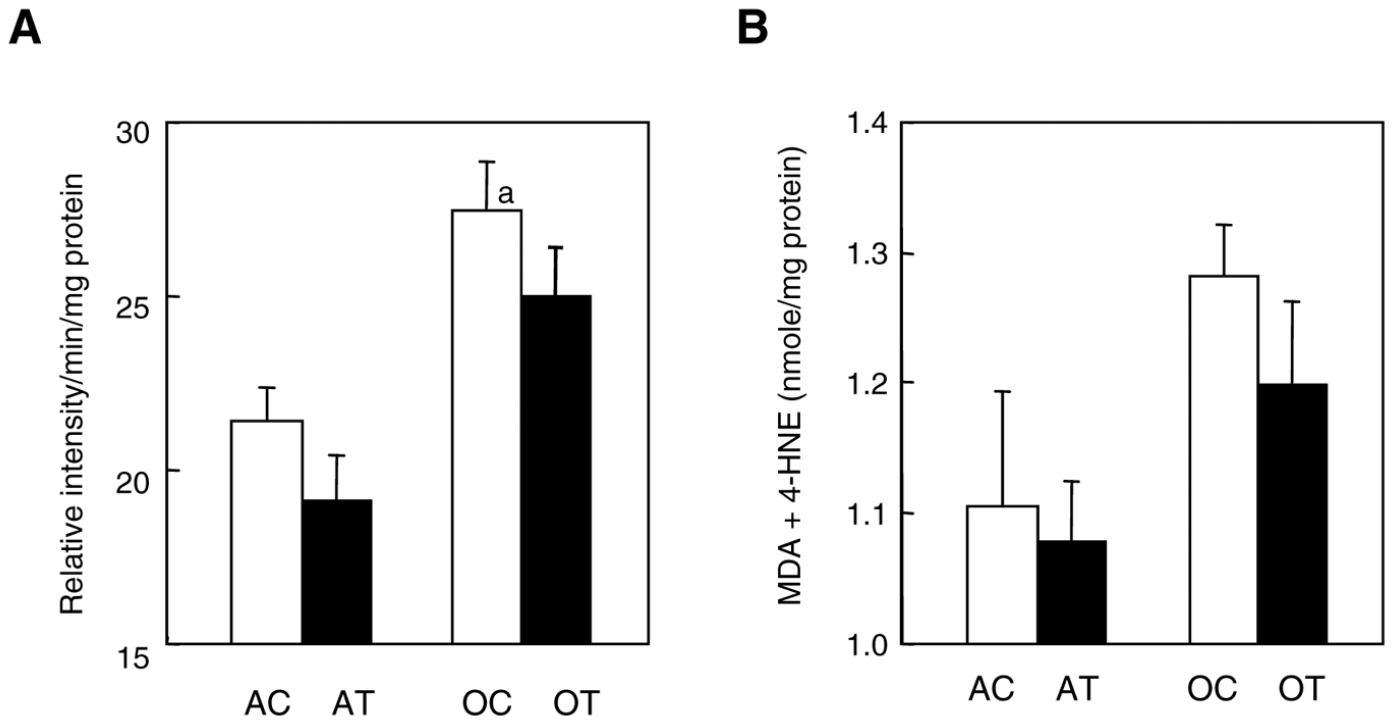

Figure 1. Exercise suppresses age-related increases of total ROS (*A*) and production of lipid peroxidation (*B*). *A*) DCF-DA method was used to determine the age/exercise effects on ROS generation in liver homogenates. ***B*)** MDA and 4-HNE were determined by using the Bioxytech LPO-586 kit. AC: adult contol, AT: adult exercise, OC: old contol, OT: old exercise. Statistical significance: a) $P < 0.01$, adult contol vs. old control group.

Fig. 2

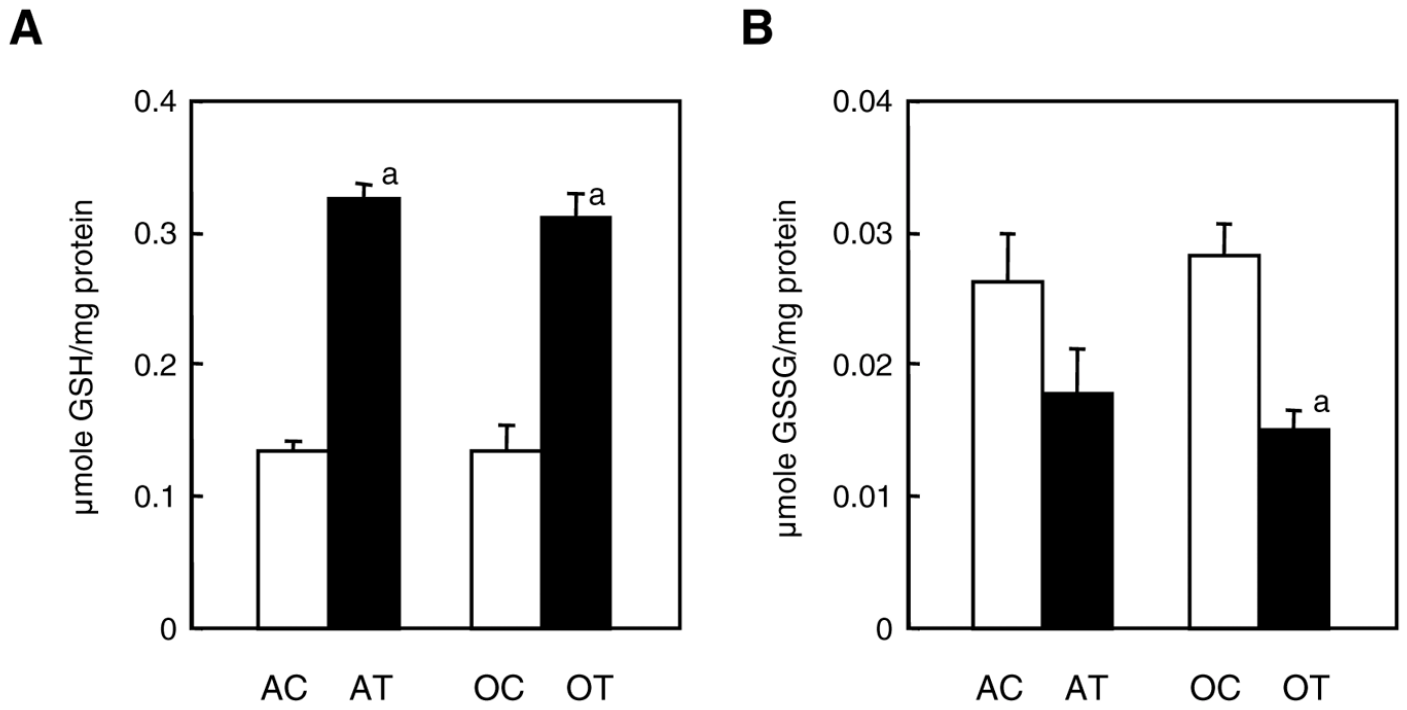

Figure 2. Effect of age and exercise on GSH/GSSG levels (A, B). GSH and GSSG contents in liver homogenates were determined as described in the Materials and Methods. Statistical significance: ***A***) $P<0.05$, between each set of aged-matched control and trained groups.

Fig. 3

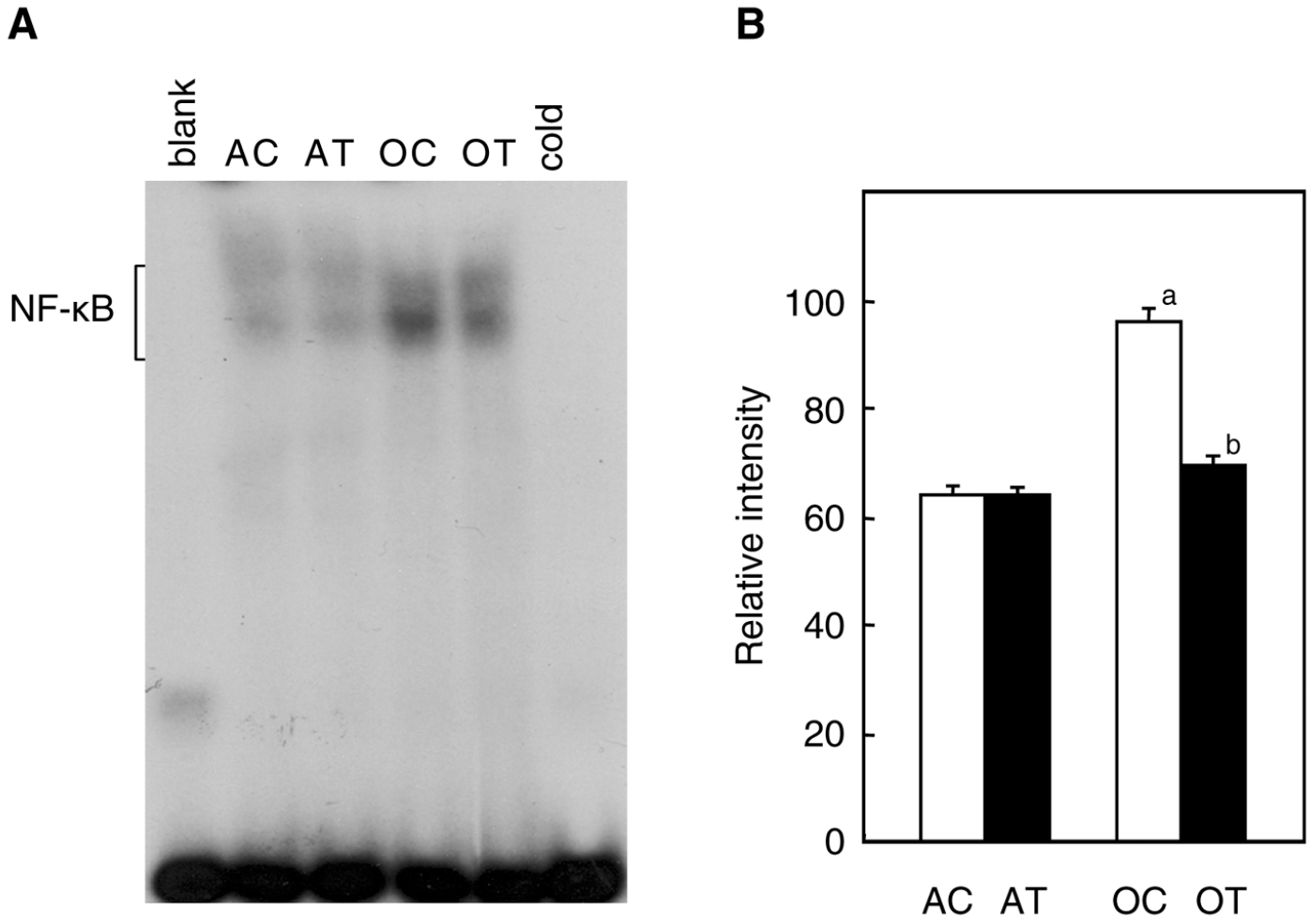

Figure 3. Exercise suppresses age-related increase in NF-κB activity. The EMSA was used to compare nuclear NF-κB binding activities in liver nuclear protein from adult and old groups correlated to exercised group. One representative result is shown from three experiments that yielded similar results. The level of NF-κB DNA binding was quantified by densitometry. The data shown are presented as arbitrary values of density. Statistical significance: a) $P<0.001$, adult contol vs. old control group; b) $P<0.001$, old control vs. old trained group.

Fig. 4

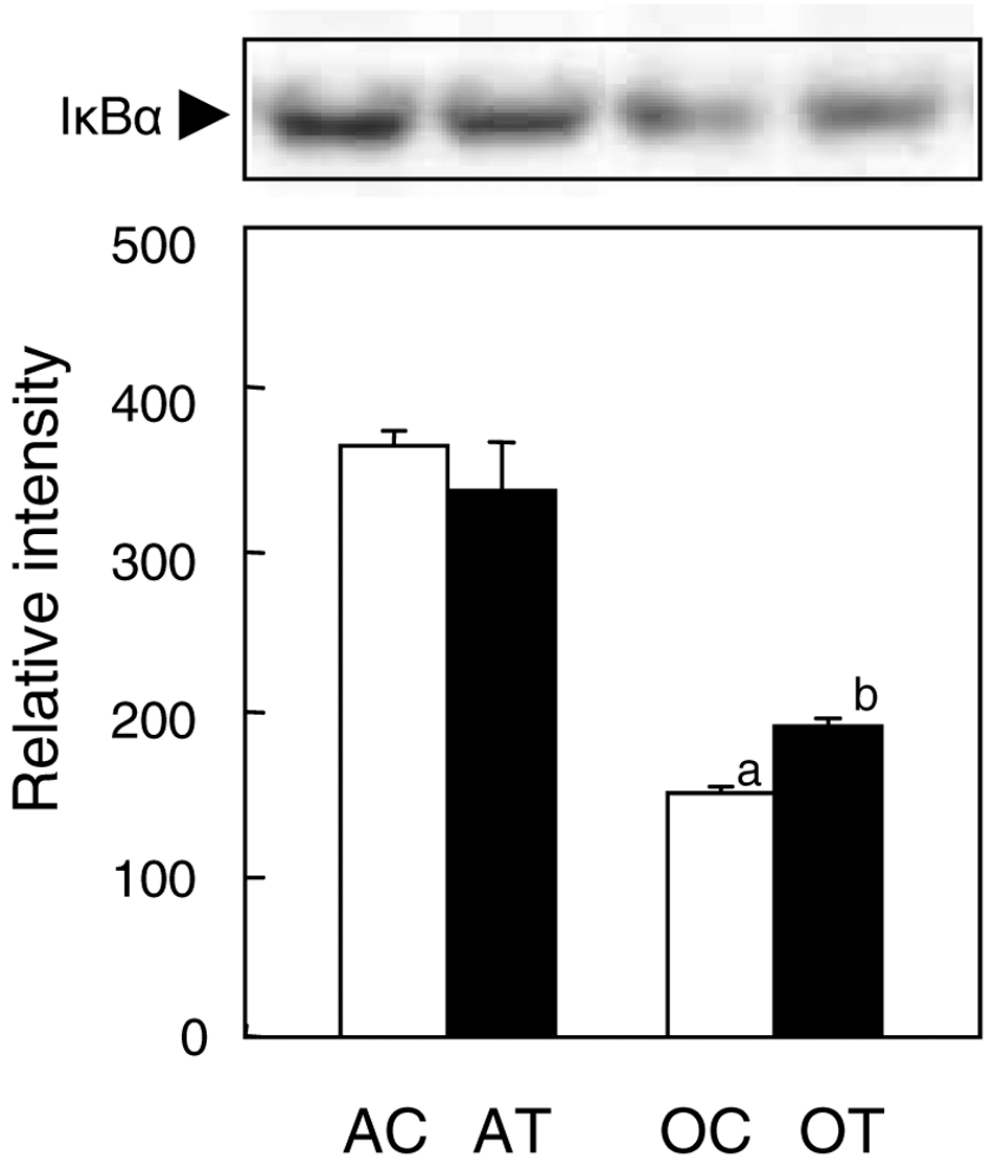

Figure 4. Exercise prevents age-related degradation of I-κBα. Western blot analysis was performed to detect hepatic I-κBα in cytosol from adult and old rats. The blots were quantified by densitometry. The data shown are presented as arbitrary values of density. Statistical significance: a) $P<0.001$, adult conrol vs. old control; b) $P<0.05$, old control vs. old trained group.

Fig. 5

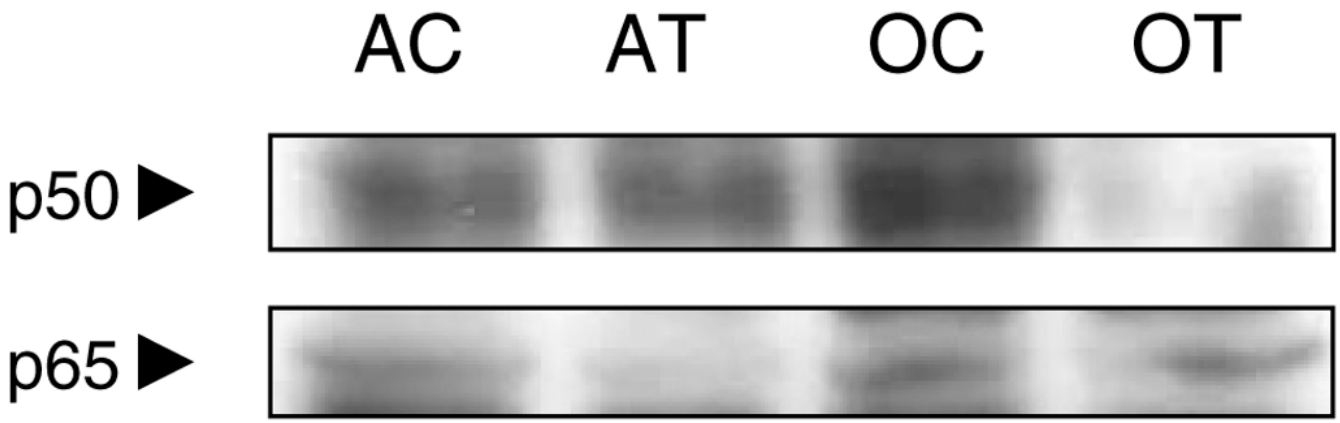

Figure 5. Effect of age and exercise on nuclear translocation of NF-κB. Western blot analysis was performed to investigate the nuclear localization of NF-κB subunits p50 and p65. Represetative results shown suggest that the amount is reduced by exercise in both adult and old groups.