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Original Research Communication

Characterization of Aging-Associated Up-Regulation of Constitutive Nuclear Factor-κB Binding Activity

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ABSTRACT

Changes occur in gene expression during aging *in vivo* and in replicative senescence *in vitro*, suggesting that aging can affect gene regulation. We have recently observed age-related changes in ubiquitously expressed, oxidative stress-responsive nuclear factor-κB (NF-κB) pathway during aging. Here we report a significant age-related increase in nuclear NF-κB binding activity together with increased protein levels of p52 and p65 components in rat liver. An additional, higher molecular weight protein band seen in their western blots suggests that their post-translational modification (but not phosphorylation) occurs in liver, which might affect their nuclear localization and binding activity during aging. However, aging did not affect the protein levels of the main IκB inhibitors (IκBα and IκBβ) or IκB kinase (IKK)-complex subunits (IKKα, -β, and -γ) involved in NF-κB activation. In addition, the level of Ser^{32}-phosphorylated IκBα was unaffected by age, suggesting that neither the IKK complex nor altered level of the main inhibitors is involved in the observed up-regulation of NF-κB binding activity. Furthermore, the expression of NF-κB mRNAs (*p50, p52, p65,* and *c-rel*) and the mRNAs of their inhibitors (IκBα and IκBβ) did not show any statistically significant age-related changes. These results indicate that the expression level of NF-κB genes is not significantly affected by aging. The up-regulation of constitutive nuclear NF-κB binding activity and increased levels of nuclear p52 and p65 proteins might affect the expression of some NF-κB target genes in the aging liver. Antioxid. Redox Signal. 3, 147–156.

INTRODUCTION

THE NUCLEAR FACTOR-κB (NF-κB) pathway is an important stress-responsive system in mammalian cells and functions as a key regulator of many defensive response genes especially in stress, inflammation, and injury responses (8, 24). In addition, NF-κB is involved in antiapoptotic responses (18, 37), which are suggested to be involved in the development of age-related, deleterious diseases (32). We have previously shown that NF-κB binding activity increases significantly with age in many mouse and rat tissues (10, 11, 13). The aging-associated changes in the NF-κB pathway may thus have profound effects on the efficiency of gene expression during defensive responses, stress, and apoptosis resistance, and maintaining the normal cellular homeostasis in aging tissues.

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The mammalian NF-κB gene family consists of five members (*p50/p105, p52/p100, p65, c-rel,* and *relB*) (1, 24), all containing an ~300-amino-acid-long Rel homology domain (RHD) at their N-terminus (24). The RHD is required for their dimerization, interactions with inhibitor proteins, nuclear translocation, and sequence-specific binding to κB sites in DNA. The functional form of NF-κB is a dimer formed of diverse combinations of these family members.

In normal resting cells, NF-κB is inactive and sequestered in the cytosol via noncovalent interactions with the inhibitor proteins, IκBs (9). An IκB monomer binds to NF-κB dimer masking its nuclear localization signal and DNA-binding domain. Mammalian IκB proteins form an IκB gene family and are structurally and functionally related. Typically, all IκB proteins contain multiple copies of the ankyrin repeats (3, 21), which interact with the RHD of NF-κB protein.

A large variety of stimuli can activate NF-κB, including cytokines, mitogens, oxidative stress, UV radiation, and various microbes and their products (e.g., 23, 24). Signals activating NF-κB target IκB proteins, although their diverse signal transduction pathways are only partially known today. Activation signals lead generally to the activation of an IκB kinase (IKK) complex (6, 20, 35), which is formed of two catalytic subunits (IKKα and IKKβ) and two regulatory subunits (IKKγ). IKK complex is responsible for the site-specific phosphorylation of IκB proteins (36) triggering their ubiquitination and degradation by the 26S proteasome pathway (22). The released NF-κB then translocates to the nucleus and binds to κB sites in its target gene regulatory regions influencing their expression. Various NF-κB dimers may exhibit distinct preferences for binding sites (1) or some accessory factors may influence their binding to cognate DNA, such as HMG1(Y) (HMG, high mobility group) protein (38).

Few direct upstream activators of IKK complex have been identified. These include NF-κB-inducing kinase (NIK) (33) and MEKK-1 (34). However, in certain activation circumstances, NF-κB activation does not involve the IKK complex or even IκB degradation (12, 14, 16, 25).

In this study, we report a significant, age-related increase in the protein levels of p52 and p65 subunits of NF-κB together with increased nuclear NF-κB binding activity in rat liver. Cytoplasmic protein levels of the main NF-κB inhibitors, IκBα and IκBβ, and the protein levels of activating IKK-complex components, however, were not affected by age. These results suggest that the IKK complex and enhanced degradation of the main IκB proteins are not involved in an aging-associated increase in nuclear NF-κB binding activity

MATERIALS AND METHODS

Animals

Wistar rats (both sexes) were obtained from the National Laboratory Animal Center (Kuopio, Finland). They represented two age categories: young rats, 3–7 months old; and old rats, 26–30 months old. Animals were killed using CO_2. Livers were removed, washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at −80°C.

Reagents

The following antibodies were used in supershift electrophoretic mobility shift assay (EMSA) and in western blotting: anti-NF-κB, p65 subunit (Boehringer Mannheim), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (Cappel), PhosphoPlus IκBα (Ser^{32}) antibody kit (catalogue no. 9240; New England BioLabs), and from Santa Cruz; p50 (NLS, X), p52 (C-5, K-27X), c-Rel (NX, CX), IκBα/MAD-3 (C-15), IκBβ) (C-20), IKKα (H-744), IKKβ (H-470), IKKγ (FL-419), and NIK (H-248). Oligonucleotides used in EMSA were consensus and mutated NF-κB, consensus and mutated Sp-1, and consensus and mutated AP-1 (Promega).

Isolation of proteins for EMSA and western blot assays

Nuclear and cytoplasmic proteins were released and purified from samples according to the modified protocol of Dignam *et al.* (7) described in more detail by Helenius *et al.* (10).

EMSA assays

The double-stranded oligonucleotide probes used in EMSA were end-labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Promega) according to the protocol of the manufacturer.

Nuclear protein samples (10 μg) were incubated (15 min, room temperature) with labeled probe (20,000 cpm). The concentration of salts was balanced by filling all samples to the same volume with the low-salt/high-salt (2:1) buffer used in protein isolation. Nonspecific binding was blocked by poly(dI/dC) (Pharmacia; 3 μg per assay of 20 μl). Nonidet P-40 (10%; BDH Chemicals) was also added. The reaction was stopped using DNA loading buffer. The bound and unbound probes were separated in native 4% acrylamide gel electrophoresis, the gel was dried on Whatman paper (3MM), and results were visualized on either an autoradiography film (Fuji) or Storm 860 PhosphoImager (Molecular Dynamics).

The protein components in specific NF-κB complex were identified using the supershift assay. After the 10-min binding reaction, a specific primary antibody against different components of the NF-κB complex was added, the mixture was incubated further (30 min, 4°C), and a normal gel retardation assay and visualization were performed (10). Supershift assays were also repeated with the treatment of nuclear proteins for 60 min in ice prior to the DNA binding reaction. Nonidet P-40 was omitted in supershift assays, and poly(dI/dC) was added at the concentration of 2 μg per assay of 20-μl volume.

UV crosslinking

UV-crosslinking technique was used to characterize the proteins that bind to the κB binding site. Assays were performed as described earlier (10).

Western blot assays

Nuclear (15 μg) and cytoplasmic proteins (20 μg) were resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P (Millipore) membrane using a semidry transfer technique (0.8 mA/cm^2, 1 h, room temperature; Pharmacia LKB Multiphor II).

Membranes were blocked prior to the immunostaining with 5% nonfat milk powder, 0.1% Tween in phosphate-buffered saline (PBS; overnight at 4°C). After that, membranes were incubated with a primary antibody (1:200 dilution in PBS, 5% fat-free milk, 0.1% Tween) for 2 h at room temperature, followed by washing (3×5 min) and incubation with a secondary antibody, either HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG (1:2,000 dilution in PBS, 5% fat-free milk, 0.1% Tween), for 1 h at room temperature. After washing (4×5 min), bound antibodies were detected on autoradiography film (Fuji) using ECL-western blot chemiluminescence reagents according to the protocol of the manufacturer (Pierce).

Northern hybridization

Total RNA was isolated from rat livers using TRIzol (GibcoBRL) reagent and the protocol of the company. The poly(A)$^+$ mRNA was further purified with PolyATract (Promega). The PCR primers used were designed with Primer Detective 1.01 software (Clontech). Gene-specific fragments for riboprobes were generated by PCR, cloned into pGEM-T Easy vector (Promega), and verified by sequencing. The plasmids and detailed information on primer sequences and PCR conditions are available upon request. The ^{32}P-labeled riboprobes were generated with the Strip-EZ kit (Ambion) and used without further purification. Rat liver mRNA (300 ng) was separated in agarose gel electrophoresis (3.3 V/cm voltage gradient, 2.5 h), transferred to nylon membrane (Magna Charge, MSI) by downward capillary process, and fixed by UV crosslinking (72 mJ/cm^2; Stratalinker 1800, Stratagene).

Hybridizations were performed in modified "high-stringency" Church buffer (4) at 55–60°C depending on the probe length and GC content. After that, the filters were rinsed once [1 × saline–sodium citrate (SSC)] and washed (1 × SSC, 0.2% SDS, 68°C, 30 min and 0.1 × SSC, 0.2% SDS, 68°C, 1 h), and signals were visual-

ized on Storm 860 PhosphoImager (Molecular Dynamics) after 1–5 days of exposure. To choose the appropriate internal standard mRNA, which could be used as a loading control, the three most widely used "housekeeping" genes were tested. After stripping with the Strip-EZ kit (Ambion), filters were consecutively reprobed with ^{32}P-labeled antisense riboprobes specific for glyceraldehyde-3-phosphate dehydrogenase, β-actin, and cyclophilin genes. Pixel volumes of specific bands were calculated with ImageQuaNT 4.2 software (Molecular Dynamics) with no background correction. For our particular aging model, cyclophilin was found to be the most invariant with age and used for standardization. The intensities of specific NF-κB bands were divided by the intensities of the cyclophilin bands of corresponding lanes, and then each ratio in a particular blot was divided by the average calculated from all samples of the same blot.

Statistics

Results were statistically analyzed with SPSS for Windows 9.0.1 using one-way ANOVA.

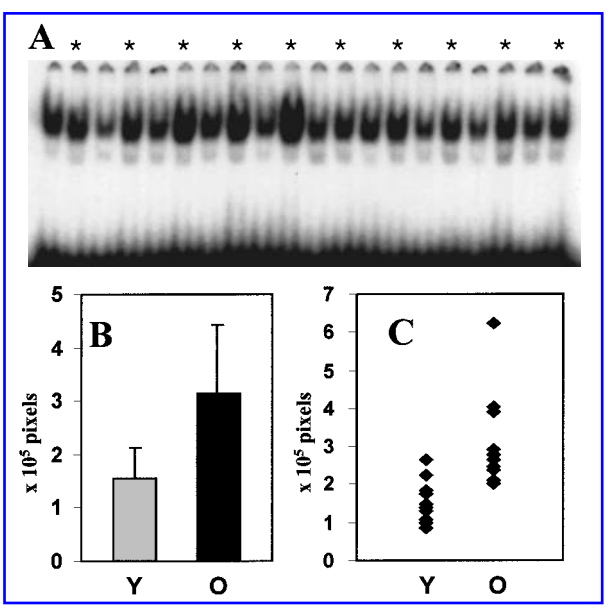

FIG. 1. Nuclear NF-κB binding activity is constitutively increased in old rat livers. (**A**) EMSA results. Old samples are marked with asterisks. (**B**) Storm PhosphoImager calculation of pixel values (means ± SD) of specific NF-κB binding. (**C**) Scatter blot of the observations. Y, young rats ($n = 10$); O, old rats ($n = 10$). The difference between Y and O is statistically significant ($p < 0.01$).

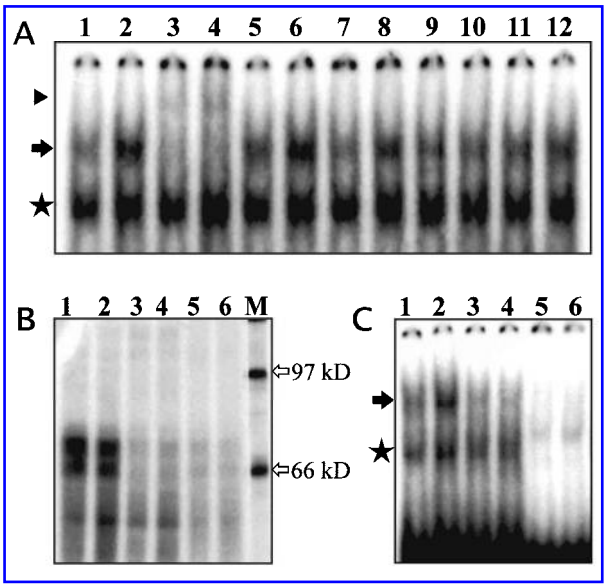

FIG. 2. Characterization of NF-κB binding activity. (**A**) Supershift assay without specific antibodies (lanes 1 and 2), with p50 antibody (lanes 3 and 4), with p52 antibody (lanes 5 and 6), with p65 antibody (lanes 7 and 8), with both p52 and p65 antibodies (lanes 9 and 10), and with c-Rel antibody (lanes 11 and 12). Even numbers show old rats. (**B**) UV-crosslinking results. Lanes 1 and 2 represent young and old liver samples UV-crosslinked with specific labeled NF-κB probe and resolved in 10% SDS-PAGE gel. Lanes 3 and 4, respectively, show the DNA binding in reactions added with 100× concentration of competing unlabeled NF-κB oligonucleotides. Lanes 5 and 6, respectively, show the DNA binding to mutated NF-κB probe in the samples of young and old liver. (**C**) EMSA assay of samples from young (lane 1) and old (lane 2) liver. Lanes 3 and 4, respectively, show NF-κB DNA binding with 50× concentration of competing unlabeled NF-κB oligonucleotide. The decrease in specific NF-κB binding activity was 53% for old rat liver and 42% for young one. On the contrary, changes in unspecific binding were 13% and 0%, respectively. Lanes 5 and 6 show DNA binding of young and old liver samples to the mutated NF-κB oligonucleotide binding site. The arrow shows the specific NF-κB complex, the arrowhead shows the supershifted complex, and the star shows an unspecific binding.

RESULTS

Constitutive increase in nuclear NF-κB binding activation with age

Aging induced a strong increase (~100%) in constitutive nuclear NF-κB binding activity in rat liver (Fig. 1), which appeared both in males and in females. These results are consistent with our previous observations of the similar age-related increase in nuclear NF-κB binding activity in many mouse and rat tissues (10, 11, 13). Supershift assay showed that the nuclear NF-κB complex contained p50 and p65 sub-

units in rat liver (Fig. 2A). Shifted bands were faint probably due to the posttranslational protein modifications (see western results). The same subunits were also observed to form nuclear NF-κB complexes in mouse heart (10).

UV-crosslinking technique was used to characterize proteins, which bind to NF-κB oligonucleotides. Figure 2B shows the three major bands over 66 kDa, which probably represent the complexes of NF-κB proteins bound with labeled oligonucleotides. Posttranslational modifications may affect the size of binding complexes (see western results). Addition of unlabeled consensus oligonucleotides reduced the specific binding (Fig. 2B, lanes 3 and 4). The mutated NF-κB binding site did not show any DNA binding in UV-crosslinking assays (Fig. 2B, lanes 5 and 6). Figure 2C confirms the UV-crosslinking results and shows that the addition of competing unlabeled consensus oligonucleotides of NF-κB reduced the specific binding to the labeled probe. Furthermore, the mutation of the specific NF-κB binding site abolished the specific binding in EMSA assay (Fig. 2C, lanes 5 and 6).

Protein levels of p52 and p65 components of NF-κB increase with age, but protein levels of the main cytoplasmic NF-κB inhibitors remain unaffected

The increased nuclear NF-κB binding activity may involve changes in protein components forming the NF-κB dimer. These possible age-related changes in the protein levels of NF-κB subunits were studied using western blotting. In aging liver, protein levels of p52 and p65 subunits of NF-κB increased significantly in both cytosolic and nuclear fractions (Fig. 3). Furthermore, an additional, higher molecular weight band was detected in all samples and in both of these proteins. However, this protein band was not due to the phosphorylation of these proteins, because the treatment of samples with calf intestine alkaline phosphatase was not able to remove this band (data not shown). On the contrary, the level of the p50 subunit was not affected by age (Fig. 3). The protein level of nuclear c-Rel showed a high interindividual variance, and the cytoplasmic level of c-Rel was unaffected by age (Fig. 3).

During activation of NF-κB, inhibitory IκBs are usually degraded, which releases the NF-κB, allowing its translocation to the nucleus. Surprisingly, aging did not affect the protein levels of the main cytoplasmic NF-κB inhibitors (Fig. 4). The protein level of IκBα showed no change with age either in cytosol or in nucleus. In addition, the protein level of IκBβ was unaffected in cytosol, but was decreased in the nucleus of aged animals. Aging did not affect the level of the Ser^{32}-phosphorylated form of IκBα, which generally precedes NF-κB nuclear translocation and activation (Fig. 4).

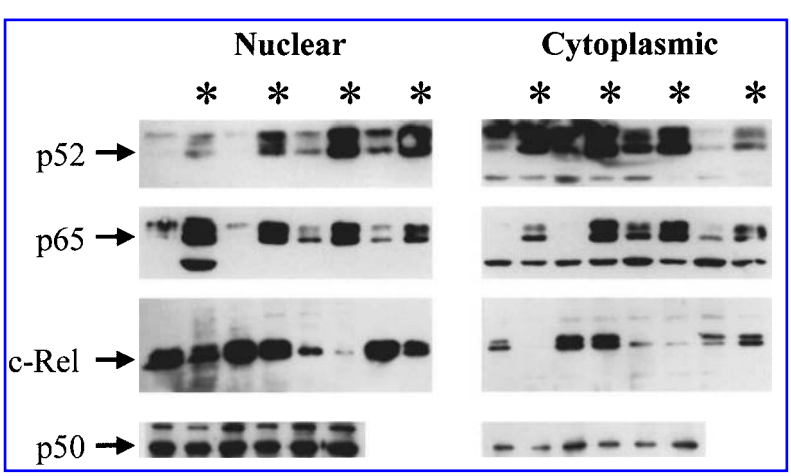

FIG. 3. Changes in protein levels of NF-κB proteins p50, p52, p65, and c-Rel during aging. The figure shows the protein levels in both nuclear and cytoplasmic fractions in the livers of young and old (lanes marked with asterisks) rats. Arrows show the specific protein band.

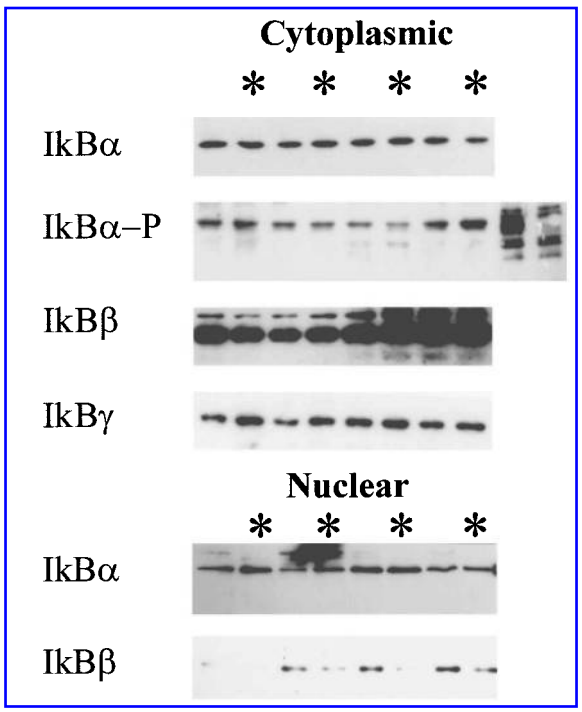

FIG. 4. Protein levels of inhibitory IκB proteins. Western blot shows the protein levels of IκBα, IκBα-P (phospho), IκBβ, and IκBγ in cytoplasm and those of IκBα and IκBβ in the nuclear fraction in the livers of young and old rats. Asterisks mark the liver samples from old rats. The IκBα-P panel shows the positive and negative control sample on right (the antibody kit from New England BioLabs; see Reagents).

IKK complex showed no age-related change

Most of the known inducers activate NF-κB via the IKK complex. The IKK complex activates the cytoplasmic NF-κB by site-directed phosphorylation of IκB inhibitors triggering their degradation, and thus releasing NF-κB. We studied the effect of aging on the main protein components of the functional IKK complex. The protein levels of IKKα, IKKβ, and IKKγ were not affected by age either in cytosol or in nucleus (Fig. 5). Interestingly, IKKβ was mainly located in nuclear fractions and was almost totally absent from cytosolic fractions in rat livers, in both young and old animals.

The activity of the IKK complex is regulated, at least in part, by phosphorylation (17, 19, 33). The IKKγ subunits have been reported to be the regulatory component of the IKK complex connecting IKK to its upstream activators. Aging may induce changes in activators of the IKK complex, and this might be involved in the increase seen in nuclear NF-κB binding activity of aged animals. A few direct upstream activators of IKK complex have been identified. Here we studied one of them, NIK (NF-κB inducing kinase). However, there were no

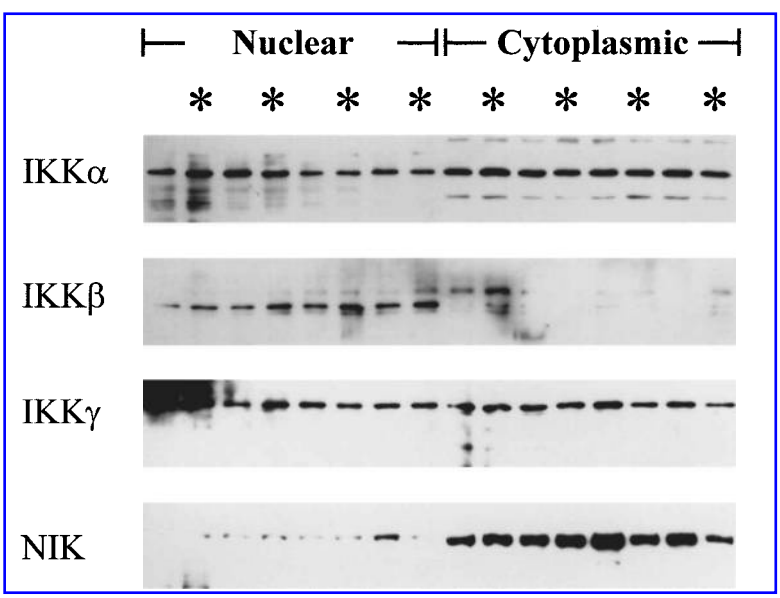

FIG. 5. Protein levels of IKK-complex subunits and NIK kinase. Western blot shows the protein levels in both the cytoplasmic and nuclear fractions from livers of young and old (lanes marked with asterisks) rats.

age-related changes in the protein level of NIK (Fig. 5).

Aging does not affect mRNA levels of NF-κB and the main IκBs

To verify the possible involvement of age-related changes in the expression level of NF-κB genes, northern hybridizations were performed. They showed that the mRNA levels of *p50, p52,* and *p65* components were slightly higher in older rats, but there were no statistically significant age-related changes in their mRNA levels (Fig. 6). In addition, the mRNA level of *c-rel* was unaffected by age (Fig. 6). Furthermore, the mRNA levels of the main inhibitors (IκBα, and IκBβ) did not show any significant change with age in rat liver (Fig. 6).

DISCUSSION

The molecular mechanisms controlling aging are still largely unknown despite the many theoretical approaches used to study these mechanisms (see reviews, e.g., 5). Oxidative stress and changes in stress resistance have been suggested to be among the major contributors to the aging process in animal tissues (26). NF-κB is one of the key regulators of the cellular responses to oxidative stress in mammalian cells (see 23, 24). In addition, NF-κB is involved in apoptotic responses in cells. Interestingly, cells are reported to become resistant to apoptosis during aging (31). Aging-associated changes in NF-κB signaling may, thus, have profound effects on maintaining cellular homeostasis in aging tissues during defensive responses, stress, and induction of apoptosis.

We have previously reported a significant up-regulation of constitutive nuclear NF-κB binding activity with age in several mouse and rat tissues (10, 11, 13). The age-related up-regulation of NF-κB binding activity has been verified by other researchers, e.g., in brain (29), liver (28, 30), and spleen (27). Here we studied in more detail these aging-associated changes in the activation of the NF-κB complex and the expression of NF-κB genes in rat liver.

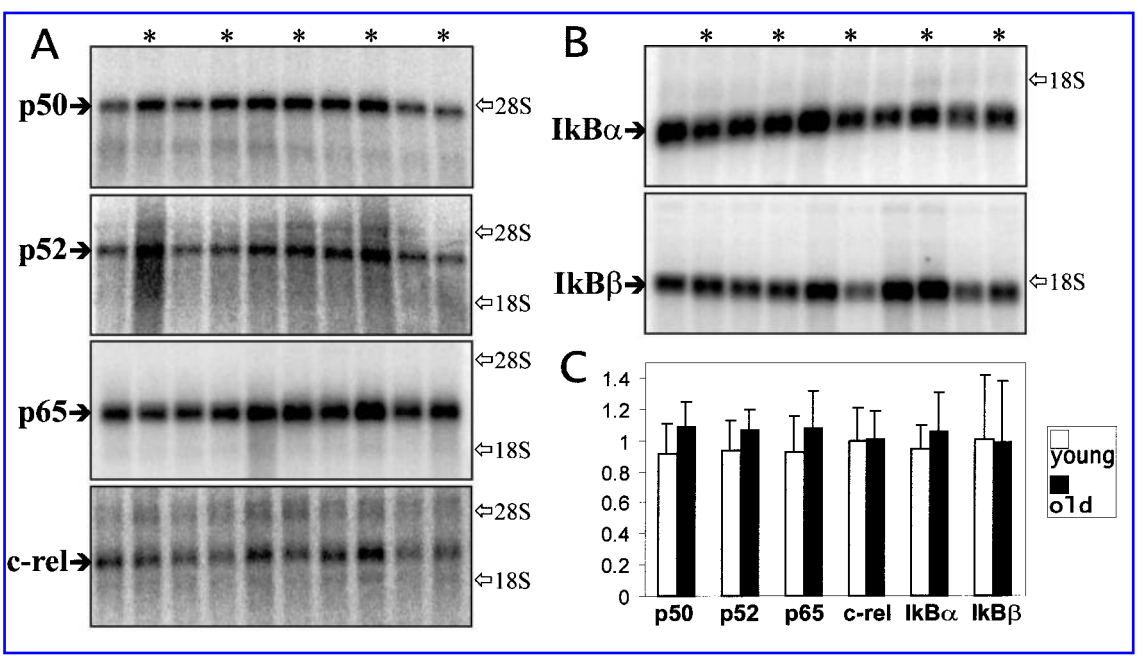

FIG. 6. mRNA expression levels of different members of NF-κB and IκB genes in the livers of young and old rats. (**A**) Northern hybridization shows the expression levels of *p50, p52, p65,* and *c-rel* in young and old rats. Old rats are marked with asterisks. The size of 18S and 28S has been marked. (**B**) Expression levels of *IκBα* and *IκBβ* genes in young and old rats (old rats marked with asterisks). (**C**) Calculation of Storm PhosphoImager values (means ± SD). The expression level of cyclophilin was used for the normalization of mRNA samples (see Materials and Methods).

In rat liver, nuclear NF-κB binding activity increases significantly with age as observed earlier (11, 28, 30). Supershift assays demonstrated that the DNA-bound NF-κB complex contained p50 and p65 proteins in rat liver, as we have previously observed in mouse heart (10). Western blot assays showed that the levels of p52 and p65 proteins were clearly increased in both cytosol and nucleus of aged livers. On the contrary, the protein level of p50 did not change with age in either cytosol or nucleus in agreement with our previous observations in other rat and mouse tissues (10, 11, 13). The nuclear level of c-Rel protein varied extensively between individual samples, but in cytoplasm the level of c-Rel was unaffected by age. Northern hybridizations further showed that the expression of the NF-κB genes studied (*p50, p52, p65,* and *c-rel*) was not affected by age, despite the fact that many of them are autoregulated by NF-κB.

We observed an additional, higher molecular weight protein band in the western assays of p52 and p65 subunits, which could be a posttranslationally modified form of these proteins. Possible modifications could be phosphorylation, ubiquitination, or acetylation. Treatment of samples with calf intestine alkaline phosphatase was unable to remove this additional band, suggesting that it is not a phosphorylated form of these proteins and furthermore that the phosphorylation level of p52 and p65 proteins was not changed with age. However, this modification of the p52 and p65 proteins may affect their half-life, nuclear localization, or DNA binding activity in cells and thus induce the observed age-related increase in nuclear NF-κB binding activity. Accessory factors, such as HMG1(Y) (38), which can enhance NF-κB binding to DNA and which also might be affected by age, may be involved in the observed increase in nuclear NF-κB binding activity.

Part of the increased level of nuclear p52 subunit in aged livers may also be due to the enhanced signals that induce processing of the cytosolic precursor, p100, to mature p52 protein capable of nuclear translocation and DNA binding. Regulation of p100 processing does not require phosphorylation. Instead, it is directed by structural determinants in RHD and in the glycine-rich hinge (2). Upstream signaling cascades leading selectively to the degradation of p100 could also be affected by age. However, these signaling pathways are still mainly unidentified.

Modifications of NF-κB do not usually lead to its nuclear translocation. This requires the detachment of cytoplasmic NF-κB from IκB inhibitors. Thus, mechanisms that control activation signals and nuclear translocation of NF-κB proteins are also possibly involved in this upregulation. We studied the protein levels of the main IκB inhibitors (IκBα and IκBβ), but they did not show any aging-associated changes. In addition, the Ser^{32} phosphorylation of IκBα was not affected in old samples. These results suggest that the aging-associated increase in nuclear NF-κB binding activity and protein levels is not due to the decreased protein level of their cytoplasmic inhibitors or reduced phosphorylation of these IκB proteins, required for their degradation. Part of the increased nuclear NF-κB binding activity might be due to the lower nuclear level of IκBβ detected in aged animals. This might be unable to remove the DNA-bound NF-κB complexes as effectively as is the case in young animals.

The protein levels of the components of the IKK complex (IKKα, IKKβ, and IKKγ) and its direct upstream activator, NIK, which transmits activation signals to NF-κB from diverse inducers (17, 19), were also unaffected by age, suggesting that they are not involved in the upregulation of NF-κB signaling with age. However, their catalytic activity was not studied, but the unaltered Ser^{32} phosphorylation level of IκBα suggests that it is not affected.

In summary, our results indicate that the aging-associated increase in nuclear NF-κB binding activity is due, at least partly, to the increased protein levels of p52 and p65 subunits with age, but unaccompanied by changes in their mRNA expression levels. An additional protein band seen in their western blots suggests that some modification of these proteins occurs in liver, which could affect either their nuclear location or their binding activity. Our results further indicate that neither the IKK complex nor enhanced degradation of main IκB inhibitors is involved in the up-regulation of nuclear NF-κB binding activity and the in-

creased nuclear level of p52 and p65 subunits in livers of aged animals.

ACKNOWLEDGMENTS

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ABBREVIATIONS

EMSA, electrophoretic mobility shift assay; HMG, high mobility group; HRP, horseradish peroxidase; IκB, inhibitor of NF-κB; IKK, IκB kinase; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; PBS, phosphate-buffered saline; RHD, Rel homology domain; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSC, saline–sodium citrate.

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