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Merja Helenius

Aging-associated Changes in NF-kappa B Signaling

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UNIVERSITY OF JYVÄSKYLÄ

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ABSTRACT

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All eukaryotes age, although the speed of the process varies between species. A variety of age-related changes in cell structures and functions have been described. However, the molecular basis of the control mechanisms of aging remain largely unknown, even though there have been many theories advanced. Aging involves alterations in gene transcription suggesting that changes in transcription factor regulation occur during aging.

We studied aging-associated changes in the NF-κB signaling pathway, which we observed to be affected by age. NF-κB is a ubiquitously expressed, inducible transcription factor in mammalian cells. It is composed of dimers of NF-κB family proteins. It functions as a key regulator of defensive responses to various environmental challenges including oxidative stress, radiation, and microbial insults. It is also involved in anti-apoptotic responses and transformation, both processes known to be associated with aging.

The results from this study show that aging up-regulates constitutive nuclear NF-κB binding activity in tissues. It induces tissue-specific changes in the protein levels of NF-κB subunits, increasing the levels of p52 and p65 proteins in liver and nuclear p52 in heart, but neither of these proteins were elevated in brain. These results indicate tissues-specific changes in the regulation of NF-κB signaling during aging. Surprisingly, the level of main inhibitor proteins, IκBα and IκBβ, and Ser-32-phosphorylation of IκBα was not changed with age suggesting that the IKK-complex is not involved. Replicative senescence attenuates transient NF-κB activation pathway and causes down-regulation of NF-κB-driven reporter genes after UVB induction, indicating that stress resistance may become reduced during cellular senescence. Our results further show that age-related changes in NF-κB signaling are differentially regulated during aging *in vivo* and *in vitro*. This limits the use of *in vitro* cell models in aging studies involving NF-κB signaling. Finally, age-related changes in NF-κB signaling in tissues are possibly involved in immune senescence and in up-regulation of apoptosis resistance in aged cells.

Key words: Aging; apoptosis; gene regulation; IκB; NF-κB; replicative senescence; transcription factor.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals:

I Helenius, M., Hänninen, M., Lehtinen, S.K. & Salminen, A. 1996. Aging-induced up-regulation of nuclear binding activities of oxidative stress responsive NF-κB transcription factor in mouse cardiac muscle. J. Mol. Cell. Cardiol. 28: 487-498.

II Korhonen, P., Helenius, M. & Salminen, A. 1997. Age-related changes in the regulation of transcription factor NF-κB in rat brain. Neurosc. Letters 225: 61-64.

III Helenius, M., Hänninen, M., Lehtinen, S.K. & Salminen, A. 1996. Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor -κB. Biochem. J. 318: 603-608.

IV Helenius, M., Kyrylenko S., Vehviläinen, P. & Salminen, A. 1999. Characterization of aging-associated upregulation of constitutive nuclear NF-κB binding activity. (Submitted)

V Helenius, M., Mäkeläinen, L. & Salminen, A.1999. Attenuation of NF-kappa B signaling response to UVB light during cellular senescence. Exp. Cell Res. 248: 194-202.

ABBREVIATIONS

aa	amino acids
ARD	ankyrin repeat domain
AP-1	activator protein-1
bp	base pair
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
cdc	cyclin dependent cyclin
cdk	cyclin dependent kinase
ck	casein kinase
CMV	cytomegalovirus
cpm	counts per minute
CPRG	chlorophenol red-β-D-galactopyranoside
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DOC	deoxycholate
ds	double stranded
DTT	dithiothreitol
EBV	Ebstain Barr virus
EGF	epidermal growth factor
E2F	adenovirus E2 promoter binding factor
EMSA	electrophoretic mobility shift assay
FGF	fibroblasts growth factor
GABA	gamma-aminobytyrate
HBV	human papilloma virus
HDF	human diploid fibroblasts
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HTLV	human T-cell leukemia virus
IAP	inhibitor of apoptosis
IgG	immunoglobulin G
IκB	inhibitor of NF-κB
IKK	IκB-kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-jun N-terminal kinase pathway
KCl	potassium chloride
kD	kilodalton
LPS	lipopolysaccharide
MEKK	mitogen-activated protein kinase / ERK kinase kinase-1
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NF-κB	nuclear factor -kappa B
NIK	NF-κB inducing kinase

NLS	nuclear localization signal
NO	nitric oxide
Oct	octamer binding protein
rpm	rounds per minute
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PEST	Proline-glutamine-serine-threonine sequence
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristate acetate
RNA	ribonucleic acid
Rb	retinoblastoma gene product
ROS	reactive oxygen species
sdi1	senescent cell derived inhibitor 1
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulfate
Ser	serine
SP-1	sequence specific transcription factor-1
SV-40	simian virus 40
TNF	tumor necrosis factor
Tyr	tyrosine
UVB	ultra violet B light
YY	ying yang

RESPONSIBILITIES OF MERJA HELENIUS IN THE ARTICLES OF THIS THESIS

Article I: Antero Salminen planned the study. I was responsible for handling the material and the data. The article was written together with Antero Salminen.

Article II: The study was planned together with Antero Salminen. The experiments were made together with Pauliina Korhonen. I performed the statistical analysis. The article was written together with Antero Salminen.

Articles III: The study was planned together with Antero Salminen. I designed and ran the experiments and performed the statistical analysis. The article was written together with Antero Salminen.

Article IV: I was mainly responsible for planning, producing and writing the study with the exception of Northern hybridizations, which were mainly made by Sergiy Kyrylenko.

Article V: The study was planned together with Antero Salminen. I designed, set up, and ran experiments with the exception that Leena Mäkeläinen gave the drug treatments. I mainly wrote the article.

1 INTRODUCTION

Humans, like all other eukaryotic organisms, age. The speed of aging varies between species, but both the physiological changes with age and the appearance of old individuals are very similar in all vertebrates. Regardless of many theoretical approaches to aging, the molecular causes of aging have remained unidentified. Oxidative stress and stress resistance are known to be among the major contributors to the aging process (Harman 1992, Sohal & Brunk 1992). Aging *in vivo* and *in vitro* involves alterations in gene transcription, which suggest that transcription factors and signal transduction pathways may be affected by age.

Transcription factors are the key elements regulating gene expression. Many of them are inducible sensors of external signals, which are mediated by a complex signal transduction network (Maniatis et al. 1987, Karin & Smeal 1992). This provides the opportunity to have multilevels of regulation steps. One of these sensors in eukaryotic cells is NF-κB (nuclear factor kappa B) pathway. It is an important stress responsive system in mammalian cells and functions as a key regulator of defensive responses to environmental challenges including oxidative stress, radiation, viruses, bacteria, and numerous chemical agents (Siebenlist et al. 1994). It is also involved in antiapoptotic responses (Liu et al. 1996, van Antwerp et al. 1996), which are thought to be involved in the development of age-related, deleterious diseases (Wang 1997).

Aging-associated changes in the NF-κB pathway may have profound effects on the efficiency of gene expression during defensive responses, stress and apoptosis resistance, and maintaining the normal cellular homeostasis in aged cells. Our purpose was to study whether aging induces changes in NF-κB signaling and compare the possible age-related changes in NF-κB factor in aging *in vivo* and in replicative senescence *in vitro*. Our results show that aging *in vivo* up-regulates constitutive nuclear NF-κB binding activity in all tissues studied. The level of increased nuclear binding activity and the observed age-related changes in NF-κB protein levels varied in different tissues indicating that there must be tissue-specific changes in the regulation of NF-κB signaling during aging. Surprisingly, the protein levels of the main inhibitors, IκBα and IκBβ, and the level of Ser-32 phosphorylated IκBα were not changed with age, suggesting that the IKK-complex is not involved in up-regulation of NF-κB

binding activity in aging tissues. Our results further show that aging *in vivo* and replicative senescence *in vitro* are differentially regulated processes. This limits the use of cultured cell models in aging studies involving the NF-κB pathway.

2 REVIEW OF THE LITERATURE

2.1 General theories of aging

Aging means a progressive loss of physiological capacities as a function of time. It culminates as an individual death. The predominant cause of death of an old individual is species-specific, but environmental factors and the genetic background are also influenced (Dice 1993, Kloeden et al. 1994). Many different theories of aging have been proposed to explain why aging has evolved (reviewed e.g. by Dice 1993). One proposes that the mechanism of aging is coupled to optimal early development; energy is saved for optimal early development, growth, and reproduction (Kirkwood 1984). In addition, aging is considered to be linked to reducing suppression of tumorgenesis, which allows young individuals to survive long enough to reproduce (Dykhuisen 1974, Sager 1991). Bell (1984) proposed that all multi-cellular organisms which have a separated germ line and somatic line undergo senescence. However Martinez and Levinton (1992) proposed, that the evolution of somatic differentiation, instead of germ-line sequestration, is needed for the evolution of senescence. A programmed cell death (apoptosis) is sometimes used as an example of the aging process, although its relationship to aging *in vivo* is unknown.

Two major theoretical approaches attempt to explain the molecular basis of cellular aging. The error theories propose that passively accumulated errors, which are the consequences of environmental insults, occur in cellular constituents (DNA, RNA, proteins, and lipids) together with an imperfect repair mechanism, during a lifetime eventually cause aging (Dice 1993). The type of damage in macromolecules, which causes the aging, and the mechanisms, by which these errors are generated, vary among the error theories. Program theories propose that aging is an active and genetically programmed process in many ways similar to early development. The common idea of program theories is that the proliferation of cells is inhibited during aging. Theories differ with regard to the changes, which cause the proliferative arrest, usually at the G1/S boundary. Some of the aging theories are summarized briefly in Table 1.

TABLE 1 Some theoretical approaches of aging.

A.	ERROR THEORIES	
Theory	Description	Reference
Cross-linking	Unfunctioning cross-linked Macromolecules	Bucala et al. 1984 Lee & Cerami 1992
Error catastrophe	Exponential errors in protein synthesis	Orgel 1963
Free radical theory	Increasing free radical reactions impair tissue functions	Harman 1956, 1992
B.	PROGRAMMED THEORIES	
Theory	Description	Reference
Telomere	Shortening telomeres as a mitotic clock	Olovnikov 1971 Reddel 1998
Terminal differentiation	Activation of death gene(s)	Dice 1993
Apoptosis resistance	Increasing apoptosis resistance impairing proper tissue function	Wang 1997

2.2 Age-related changes in cell structures, metabolism, and functions

Aging reduces physiological capacities (Dice 1993). This reduction is due to the changes in cell structures and functions in connection with the genetic background and environmental influence. The rate of this reduction varies in different tissues. Thus, the rate of aging will vary in different tissues (Goldstein 1990, Dice 1993, Masoro 1995).

There are some general aging-associated changes in the structures and functions of human cells: (1) Senescent cells are generally larger, flat and less motile (Cristofalo & Kritchevsky 1969, Mitsui & Schneider 1976, Cristofalo & Pignolo 1993). (2) Cell and nuclear size is increased (Mitsui & Scheider 1976). (3) Nucleolar size (BeMiller & Lee 1976) and the number of multinucleated cells (Matsamura 1980) are also increased. (4) Senescent cells have vacuolated cytoplasm, evacuated endoplasmic reticulum, a prominent Golgi apparatus, and large lysosomal residual bodies (Masoro 1995). (5) The synthesis rate of macromolecules (protein, DNA, RNA) decreases, while the content of macromolecules (proteins, lipids, glycogen, RNA) increases. The exception is DNA the content of which reflects the amount of DNA at the G1 stage (Goldstein 1990, Masoro 1995).

In senescent human cells, proteolysis slows down (Dice 1989, 1993) which leads to the accumulation of waste products and abnormal proteins inside the cells, e.g. lipofuscin (Furano et al. 1984). These waste products are stored mainly in residual bodies. In rats, the number and size of lysosomes increase during senescence (Dice 1993, Lakatta 1993). However, the number of mitochondria decreases with age (Sohal & Brunk 1992). In addition, mutations in human mitochondrial DNA increase during aging, which may be related both to

normal and the diseased state of aging (Kadenbach et al. 1995). The amount of filaments, especially intermediate filaments, in cellular cytoskeleton increase during aging in humans decreasing the mobility of cells (Wang 1985).

Aging increases glycation of macromolecules impairing their function. (Bucala et al. 1984, Lee & Cerami 1992). The level of oxidatively damaged, and thus unfunctioning, macromolecules, particularly proteins, also increase with age (Stadtman 1989, Tabatabaie & Floyd 1996). Both stress resistance (Rickhardson & Holbrook 1996, Kapahi et al. 1999) and general immunological responses (Coffman & Cohen 1989, Makinodan 1998) decrease during mammalian aging. In addition, marked changes occur in endocrine systems in humans (Perry 1999). Aging is accompanied by the increasing risk of degenerative, age-related diseases like atherosclerosis (Stout 1987), cardiovascular disease (Breithaupt-Grogler & Belz 1999, Crow et al. 1996), non-insulin-dependent diabetes mellitus (Armbrecht et al. 1993), osteoporosis (type II) (Armbrecht et al. 1993), cancer (e.g. Kallioniemi & Visakorpi 1996), or neurodegenerative diseases like Alzheimer's disease (Selkoe 1998).

Currently, food restriction is the only way known to increase the life span of mammals (Masoro et al. 1991). Starvation decreases the accumulation of oxidized damages in proteins and DNA (Ames & Shigenage 1992, Sohal & Brunk 1992). In animal tissues, oxidative stress has been shown to be one of the most important contributors to aging process (Adelman et al. 1988, Harman 1992, Ames et al. 1993). Aging increases the amount of free radicals which mitochondria produce e.g. in houseflies and the brain, heart and liver of rat (Sohal & Brunk 1992), thereby increasing the oxidative stress with age. ROS (reactive oxygen species), which are incompletely reduced forms of oxygen and their byproducts, are continuously being generated as byproducts of normal oxidative metabolism and as due to environmental insults (Halliwell & Gutteridge 1984, Sohal & Brunk 1992). They are extremely toxic to cells because they can attack all types of macromolecules, initiating chain reactions and inducing various types of oxidative damage (e.g. Richter et al. 1988, Stadtman 1992, Ames et al. 1993). This damage leads, in general, to an altered structure of molecules and their inactivation (Ames et al. 1993). Oxidative stress also affects gene expression (e.g. Abate et al. 1990, Schreck et al. 1992, Piette et al. 1997).

2.3 Senescence associated arrest in proliferation

Differentiated cells normally have a limited capacity to divide (Haywick & Moorehead 1961). Aging reduces this division potential (Cristofalo & Pignolo 1993). Senescence in culture, also called replicative senescence, is suggested to reflect the processes that occur in aging tissues *in vivo* (Schneider & Mitsui 1976, Goldstein 1990). Cell cycle arrest in senescent cells resembles a process of terminal differentiation (Goldstein 1990) in which the proliferation capacity is irreversibly lost.

Growth arrest associated with senescence is an undefined state where normal diploid cells both fail to complete the signaling cascades which

normally lead to S-phase and are unable to express cell cycle regulated genes normally, especially G1/S genes (Chen 1997). In replicatively senescent human diploid fibroblasts, the cell cycle is arrested at late G1, near to the G1/S boundary, which likely is a fundamentally distinct state from the G0, a quiescent state of non-proliferating young cells (Goldstein 1990, Masoro 1995). This growth arrest is accompanied by a failure to carry out early G1 signal transduction cascades, e.g. activation of PKC and induction of *c-fos*, together with loss of responsiveness to a variety of growth factors. There is also failure in the expression of late G1 genes whose products are required for S-phase entry and DNA synthesis in young, replicating cells. Senescent cells have been shown to underexpress various regulatory factors, which are needed for proper cell cycle progression. However, their possible role in senescence associated growth arrest is not known. Table 2 shows a comparison of some of these factors between senescent cells and young cells.

TABLE 2 Changes in cell cycle regulatory components in senescent cells compared to those of young cells.

Cell cycle Component	Senescent cells	Young cells	Reference
Cdc2 Mrna	Down-regulated	Expressed	Stein et al. 1991
Cyclin A Mrna	Not expressed	Cell cycle regulated	Afshari et al. 1993
Cyclin C, D, E mRNA	Expressed	Cell cycle regulated	Afshari et al. 1993
c-fos2	Not expressed	Increases at early G1	Seshadri & Campisi 1990
c-myc mRNA	Expressed	Increases at early G1	Rittling et al. 1986
P21 mRNA	Increased	Expressed	Noda et al. 1994
Cyclins A & B Proteins	Down-regulated	Cell cycle regulated	Stein et al. 1991
Cyclins D1 & E Proteins	Elevated	Expressed	Dulic et al. 1993
Cdk2 protein	Low activity	High activity	Dulic et al. 1993
Cdc2 protein	Down-regulated	Expressed	Richter et al. 1991
Retinoblastoma protein	Unphosphorylated	Phosphorylated at G1/S	Stein et al. 1990
p21 protein	Elevated	Expressed	Afshari et al. 1993
P16	Elevated	Expressed	Zhu et al. 1998
Sdi1 protein	Elevated	Expressed	Noda et al. 1994

A complex chain of events is proposed to lead to cellular senescence, which is still only partially understood. McConnell et al. (1998) suggested that the inhibition of G1-specific cdk activity is sufficient to induce senescence associated phenotypic changes in primary fibroblasts. The inability of senescent cells to phosphorylate Rb normally at the G1/S transition (Stein et al. 1990) is also thought to affect the normal cell cycle progression. One proposed mechanism involves the interaction of unphosphorylated Rb with E2F (Chellappan et al. 1991) leading to down-regulation of E2F activity (Hiebert et al. 1992). E2F is a transcription factor, which up-regulates the expression of many genes required for DNA synthesis (Mudryj et al. 1990). In addition, cdk2

kinase complex has been shown to have very low enzymatic activity in senescent cells (Dulic et al. 1993).

Cell cycle inhibitor proteins, which reversibly arrest the cell proliferation after damage, have also been postulated to be involved in the activation of the senescence associated growth arrest. Aging increases the level of p21 protein, a potent cdk inhibitor, but does not affect the level of p53 protein (Afshari et al. 1993). However, Medcalf et al. (1996) showed that p53 and p21 are not required for replicative senescence. The level of senescent cell derived inhibitor 1 (sdi1), which inhibits DNA synthesis by inhibiting multiple G1 cyclin-cdk complexes, increases significantly in senescent cells, this being closely paralleled to the onset of senescence and cell proliferation arrest (Noda et al. 1994). Mazars and Jat (1997), however, showed that the accumulation of cdk inhibitors, p21Waf1/Cip1/Sdi1, does not correlate with the finite life span of rodent embryo fibroblasts. Instead, they identified a new cdk inhibitor protein, p24, the expression of which correlated with the finite proliferation potential and whose activity may be regulated by sequestration. In addition, the Raf initiated kinase cascade can induce irreversible arrest of proliferation and the premature onset of senescence, at least partly, by inducing cyclin-dependent kinase inhibitor p16(Ink4a), the level of which is elevated in senescent cells (Zhu et al. 1998). A phosphatidylinositol 3-kinase inhibitor has also been shown to induce a growth arrest with a senescence-like phenotype (Tresini et al. 1998).

Some other proposed mechanisms, why senescent cells fail to synthesize DNA include: (1) A post-transcriptional block in the expression of the proliferating cell nuclear antigen (PCNA) which is a co-factor of DNA polymerase-δ (Chang et al. 1991). (2) A reduced expression of *c-fos* in senescent cells (Seshadri & Campisi 1990) which is require for cells to progress through the G1 phase to the S phase (Holt et al. 1986). However, Rose et al. (1992) showed later that expression of c-fos and functional AP-1 activity alone in replicative senescent HDF are not sufficient for DNA synthesis. (3) The differentially regulated intracellular Ca^{2+} concentration because, in senescent cells, calmodulin do not exhibit the cell cycle-dependent pattern seen in young cells (Brook-Frederich et al. 1993).

2.4 Changes in gene expression

Gene expression is regulated mainly at the level of transcription initiation (Dynan & Tjian 1985, Lewin 1997). During cellular aging, the rate of transcription slows down both in the nucleus (Dice 1993) and in mitochondria (Calleja et al. 1993). These age-related changes in gene expression are gene specific but generally are attributable to reduced transcription (Dice 1993, Thakur et al. 1993). The reasons for this reduction are largely unknown. The characteristic senescence-related changes in gene expression have also been shown to be highly cell-type specific (Shelton et al. 1999).

Transcription factors are the key elements in the regulation of transcription. A complex signal transduction network couples the external activating signals to transcription factors. Aging induces changes in signal transduction pathways followed by changes in the efficiency to transcribe genes. The ability of senescent cells to respond to external signals is greatly reduced (Cristofalo et al. 1989, Cristofalo & Pignolo 1993): the response of myocardium to adrenaline (Lakatta 1993), general immunological responses (Coffman & Cohen 1989), as well as the response to growth hormones (EGF and PDGF) (Gerhard et al. 1991), and heat shock (Luce and Cristofalo 1992) are all reduced in aged cells. The number of receptors on the plasma membrane collecting external signals has been shown to be unaffected by age (Finocchiaro et al. 1985, Gerhard et al. 1991, Ishigami et al. 1993). Instead, the level of phosphorylated signaling proteins is reduced in aged cells (Heydari et al. 1989, Stein et al. 1990, Armbrecht et al. 1993). A few examples of signal transduction pathways also exist which function more efficiently in senescent fibroblasts, e.g. response to PMA (De Tata et al. 1993).

Histone acetylation level, which is essential for transcriptional activation (Grunstein 1997), is reduced in senescent HDF, decreasing the rate of transcription initiation (Pochron et al. 1978). Many of the transcription factors studied show no aging associated changes but aging is known to affect Sp-1, AP-1, YY-1, YY-2, and NF-κB (Ammendola et al. 1993, Riabowol et al. 1992, Dimri & Campisi 1994, Adrian et al. 1996, Helenius et al. 1996, Walter & Sierra 1998). During aging, the DNA binding ability of transcription factor Sp-1 is greatly reduced, although its protein level is unaffected (Ammendola et al. 1993). Sp-1 may be involved in the senescence associated growth arrest, because the Sp-1 binding site is the most common cis-element found in the promoters of G1/S genes which are down-regulated in senescent cells (Pang & Chen 1994). The binding activity of transcription factor AP-1 selectively declines when cells begin to age. Its composition also changes from the normally more common Jun-Fos heterodimer (a more potent gene activator form) to the Jun homodimer (Riabowol et al. 1992). The expression of *c-fos* gene is considerably reduced in senescent cells (Seshadri & Campisi 1990).

Some researchers have also suggested that elongation during translation is the most vulnerable step in protein synthesis during aging (Lee et al. 1996). The prime candidate to be altered has been postulated to be an elongation factor 1-α (Lee et al. 1996), which is essential for translation progression and helps to bind charged tRNAs to the ribosome (Lewin 1997). Several molecular markers of replicatively senescent cells have been cloned using a variety of techniques (e.g. in Masoro 1995).

2.5 Age-related changes in major tissues

Age-related changes occur in all tissues, although the rate of change varies in different tissues. In addition to the genetic background, environmental factors

(diseases, diet, life-style etc.) have a great impact. The most prominent changes are those, which occur in cardiovascular and neuronal functions.

Several age-related changes occur in the cardiovascular system. With age, the histology of the artery vessel wall changes dramatically and this is accompanied by dilation of most large and medium-size arteries, which become thicker and less compliant (Crow et al. 1996). The collagen content in vessel walls doubles with age and marked changes also occur in elastin which density decreases (Michel et al. 1994, Fornieri et al. 1992). Smooth muscle cells of vessels seem to relocate from positions where they normally control vascular tone to regions where they cause obstruction and diseases (Crow et al. 1996). Increasing stiffness of large and medium size arteries (Avolio et al. 1985) and wall thickness with age is accompanied by an increase in arterial diameter (Cliff 1970). This leads to increased aortic pulse-wave velocity (Masoro 1995). In addition, both the systolic pulse pressure and mean arterial pressure increase with age (Masoro 1995). The aged heart exhibits left ventricular hypertrophy mainly due to a marked myocardial cell enlargement (Crow et al. 1996). The aging heart also shows many aging-associated alterations in the expression of myocyte-specific genes (Schneider & Parker 1990), increased collagen content (Crow et al. 1996), and a shift in myosin heavy chain isoforms (O'Neill et al. 1991). In addition, cardiomyocytes lose their β-adenergic responsiveness during aging (Crow et al. 1996).

Many studies have focused on aging associated changes in the maintenance and regulation of brain function. Most of the recent knowledge comes from studies of age-related neurodegenerative diseases, particularly Alzheimer's disease. In the aging brain, considerable changes occur in glutamate (a major excitatory transmitter in the central nervous system) and GABA (a major inhibitory neurotransmitter). The functionality of these systems is, however, maintained with the help of different balancing systems (Masoro 1995). Minor age-related changes have also been reported to occur in some of the neurotrophic factors e.g. FGF-1 (fibroblasts growth factor-1) (Date et al. 1990) and in the immune system (microglial) responses (Perry et al. 1993). Masoro (1995) proposed that during aging, many minor compensation processes in brain functions begin to have increasingly wider consequences until the point is reached where compensation in one system has a destructive influence on another system.

In the aging liver, adaptive responsiveness declines and reserve capacity is reduced. However, clinical tests show that liver functions are still maintained well (Schmucker 1998). Many structural studies of age-related alteration in liver show conflicting data. Aging seems to be associated with a decline in the number of mitochondria and an increase in the volume of mitochondria in liver (De la Cruz et al. 1990). A clear age-related change is seen in the increased volume of both secondary lysosomes and residual bodies and concomitant accumulation of lipofuscin (Schmucker 1990). Age-associated deficiencies also occur in various biliary functions (Schmucker 1998). Both serum and biliary cholesterol levels appear to become elevated with age, which increase the incidences of coronary disease and gallstones. The hepatic clearance of certain drugs decreases and the frequency of adverse drug reactions increases with age.

The ability to respond to various extracellular stimuli decreases in aging liver (Heydari et al. 1993). The regenerative potential of liver appears to remain intact, though the rate of regeneration does decrease with age (Rabes et al. 1969).

2.6 NF-κB signaling pathway

NF-κB is an inducible, ubiquitously expressed transcription factor identified initially in B-lymphocytes by Sen & Baltimore (1986). It is a critical regulatory pathway involved in the expression of many genes, especially those which are involved in the defensive cellular responses to stress, inflammation and injury (Siebenlist et al. 1994). It also takes part in the regulation of growth and development (Siebenlist et al. 1994, Tumang et al. 1998), inhibition of apoptosis (Van Antwerp et al. 1996, Beg & Baltimore 1996, Liu et al. 1996, Kawakami et al. 1999), transformation (Finco et al. 1997, Reuther et al. 1998), and metastasis of tumor cells (Tozawa et al. 1995).

NF-κB consists of a family of structurally related, interacting proteins which forms dimers and are localized in the cytoplasm of unstimulated cells. The mammalian NF-κB/Rel family contains five members (p105/p50, p100/p52, p65, c-rel, and relB) (Table 3A) which are members of an evolutionary conserved family of proteins found also in insects (Dorsal and Dif proteins) (Ghosh et al. 1990, Nolan et al. 1991, Meyer et al. 1991, Bours et al. 1992, Siebenlist et al. 1994 and references therein). NF-κB/Rel proteins can be grouped into two subclasses on the basis of their structure, activation, and transactivation capacity. Members of subclass one (p105/p50 and p100/p52) contain ankyrin-repeats-like structures in their C-terminal end (like their inhibitors), they are produced as inactive precursor proteins, and they are weak gene activators or function as repressors. Members belonging to the second subclass (p65, c-Rel, and RelB) contain a transactivation domain (Ballard et al. 1992, Schmitz & Baeuerle 1991). They are produced in a form of active, mature proteins, and they are potent gene activators. Theoretically, the members of this family can form almost all possible combinations of homo- or heterodimers with each other. However, only certain combinations have been detected *in vivo* (Siebenlist et al. 1994, Baldwin 1996). The classic NF-κB-complex, which is the best characterized of the complexes, is a heterodimer of p50/p65 subunits (Grimm & Baeuerle 1993).

NF-κB is maintained in the cytoplasm of normal, resting cell by its inhibitor (IκB) which tightly regulates the biological function of NF-κB. A large variety of stimuli can activate it, including cytokines, UV-radiation, oxidative stress, bacterial LPS, viruses etc. (table 4) (Siebenlist et al. 1994, Baldwin 1996). There are exceptions, for instance, mature B-lymphocytes (Sen & Baltimore 1986), some monocytes, and magrophages (Baeuerle & Henkel 1994) in which NF-κB is constitutively active. NF-κB regulates the expression of diverse genes,

including those involved in immune functions, inflammatory responses, cell adhesion, growth and death (Table 5) (Siebenlist et al. 1994, Ghosh et al. 1998).

TABLE 3 Structure and some properties of mammalian NF-κB/Rel family members and its inhibitor IκB family members (Siebenlist et al. 1994, Baldwin 1996, Ghosh et al. 1998). (mw = molecular weight, kD = kilo Dalton, ? = unknown)

A.	NF-κB/Rel Family		
Gene	Protein	Mw (kD)	Transactivation potential
nfkb1	p105/p50	105/50	none or weak
nfkb2	p100/p52	100/52	none or weak
rela	p65 (Rel-A)	65	most potent
c-rel	c-Rel	75	potent
relb	Rel-B	68	potent
B.	IκB Family		
Gene	Protein	Mw (Kd)	Inhibition preference
ikba	IκBα	37	p65, c-Rel
ikbb	IκBβ	46	p65, c-Rel
ikbe	IκBε	45	p65, c-Rel
Ikbr	IκBR	?	p50, p65
bcl-3	Bcl-3	46-56	p50, p52
nfkb1	IκBγ (C-terminal end)	70	p65, c-Rel, p50
nfkb1	p105 (precursor)	105	p50, p52
nfkb2	p100 (precursor)	100	p50, p52

2.6.1 Structure of NF-κB complex

The active DNA-binding form of NF-κB is a dimer formed of various combinations of the members of NF-κB/Rel -family. All the members of this family share a common (35-61% identical), about 300 amino acids long Rel homology domain (RHD) in their N-terminal end (Siebenlist et al. 1994). The RHD determines their dimerization, interactions with inhibitory proteins, nuclear translocation, and binding to κB elements in DNA.

RHD is formed of two immunoglobulin (Ig)-like structures (Baeuerle 1998). The N-terminal part of RHD contains an essential motif for DNA binding (Toledano et al. 1993). It makes sequence-specific contacts with the DNA major groove and it is also used as the backbone of the molecule (Baeuerle 1998). The C-terminal half of RHD facilitates dimerization (Ganchi et al. 1993) and makes contact with DNA backbones (Baeuerle 1998). Different amino acids may be involved in dimerization with different members of NF-κB gene family (Dobrzanski et al. 1994, Siebenlist et al. 1994). The C-terminal end of RHD also contains a transactivation domain in the members of subclass 2. In addition, the extreme C-terminal end of RHD contains the nuclear localization sequence (NLS) formed of conserved cluster of positively charged amino acids (Arg-Lys-Arg-Gln-Lys and Lys-Arg-Lys-Arg) (Grimm & Baeuerle 1993).

NF-κB complex binds to κB-sites (consensus GGG GAC TTT CCC) in target genes regulatory regions (Siebenlist et al. 1994). Each of the various NF-κB dimers may exhibit distinct preferences for binding sites (Baldwin 1996, Grigoriadis et al. 1996). The binding of NF-κB to the κB-site induces DNA bending which is modulated by the presence of the p65 subunit in the complex (Schreck et al. 1990, Matthews et al. 1995). The highly conserved cysteine 62 in RHD of p50 mediates a redox effect (Toledano et al. 1993) and has to be in its reduced state for binding to DNA (Matthews et al. 1992, 1993). Another, conserved amino acid, lysine 80, takes part to DNA binding and stabilizes the complex (Michalopoulos & Hay 1999). Crystal structures of DNA bound p50, p52, and p65 homodimers, and the p50/p65 heterodimer has been characterized (Ghosh et al. 1995, Müller et al. 1995, Cramer et al.1997, Huxford et al. 1998, Jacobs & Harrison 1998).

2.6.2 IκB proteins

NF-κB proteins are sequestered in the cytosol via non-covalent interactions with IκB proteins (inhibitors of NF-κB) (Siebenlist et al. 1994, Ghosh et al. 1998). The IκB monomer binds to the NF-κB dimer and prevent its nuclear translocation and DNA binding (Arenzana-Seisdedos et al. 1995). Mammalian IκB proteins (IκBα, IκBβ, IκBγ, IκBε, precursor proteins p105 and p100, IκBR, and Bcl-3) form the IκB family (Table 3B) (Gilmore & Morin 1993, Li & Nabel 1997, Whiteside et al. 1997). They are structurally and functionally related. Typically, all IκB proteins share a partially conserved ankyrin repeat domain (ARD) in their central region, which is used for binding to NF-κB proteins (Gilmore & Morin 1993). This domain harbors six to eight ankyrin motifs (Bork 1993), which each are 33-amino acid long and all contain a similar core sequence (Blank et al. 1992, Nolan & Baltimore 1992, Gilmore & Morin 1993). In addition, IκB family members have an acidic PEST region (proline-glutamine-serine-threonine sequence) regulating their basal degradation in their C-terminal end with the exception of IκBε and Bcl-3 (Haskill et al. 1991, Verma et al. 1995, Li & Nabel 1997). The N-terminal region of IκB proteins contains both phosphorylation and ubiquitination sites and regulates signal-dependent degradation (Wulczyn et al. 1998).

Ankyrin repeats mediate protein-protein interactions. The inhibition specificity of IκB is thought to reside in the number of ankyrin repeats (Thompson et al. 1995). IκBα with 5 repeats targets specifically to p65 and c-rel. In addition, IκBβ, with 6 repeats, appears to have the same kind of affinity. In contrast, both IκBγ and Bcl-3, with 7 repeats each, target specifically p50. IκBα and IκBβ are thought to be primarily responsible for regulation of the prototypical NF-κB activity because they both interact with potent trans-activators, p65 and c-rel (Thompson et al. 1995). Bcl-3 binds only nuclear p50 and p52 homodimers (Nolan et al. 1993). It contains a transactivation domain and can also function as transcription activator by forming a ternary-complex with either DNA-bound p50 or p52 homodimers (Franzoso et al. 1992, Bours et al. 1993, Lenardo & Siebenlist 1994).

The different amount of ankyrin repeats in IκBα and -β may reflect their distinct role in NF-κB activation. Both factors display similar inhibitory activities and are present in equal amounts in cells and in complexes containing similar NF-κB proteins. However, they respond to different inducers of NF-κB. All known inducers of NF-κB target IκBα inducing a fast and transient activation of NF-κB (Baldwin 1996). However, a few known inducers (LPS and IL-1) lead also to loss of IκBβ (Thompson et al. 1995). Degradation of IκBβ is phosphorylation dependent and slower than that of IκBα (DiDonato et al. 1996, McKinsey et al. 1996). It induces a persistent activation of NF-κB with a cell-type-specific difference in magnitude (Thompson et al. 1995). In addition, both NF-κB and IκB are widely expressed in different tissues but the IκBα gene is up-regulated by nuclear NF-κB (Le Bail et al. 1993) whereas IκBβ is not (Thompson et al. 1995). They are also able to dissociate NF-κB complexes bound to DNA (Zabel & Baeuerle 1990, Zabel et al. 1993) leading to another potential level of regulation.

Two types of inactive NF-κB complexes are found in the cytoplasm of cells. One consists of a mature NF-κB dimer bound to a monomeric IκB inhibitor (e.g. p50/p65 + IκB-α). The other type consists of mature NF-κB protein bound to the NF-κB precursor protein, either p105 or p100, which both can function as IκB-like proteins (e.g. p105/p65) (Rice et al. 1992, Hatada et al. 1993, Mercurio et al. 1993, Naumann et al. 1993, Scheinman et al. 1993, Dobrzanski et al. 1995). Lin et al. (1998) propose that p50 is produced using a unique co-translational processing of the amino acid chain with 26S proteasome instead of cleaving the precursor p105. This kind of control system could ensure that the production of both p50 and p105 subunits are kept in balance and enables their independent functions in gene regulation. In addition, Kanno et al. (1994) reported the existence of a third type of cytoplasmic inhibitory complex in T cells, the p50-p65 dimer, which is inhibited by p100 precursor.

The best characterized IκB protein is IκBα. The ankyrin repeats of IκBα recognize and bind to the RHD of the NF-κB complex, inhibiting its nuclear translocation and DNA-binding (Malek et al. 1998). The first two ankyrin repeats cover the α helix containing the NLS and the sixth ankyrin repeat occlude the DNA binding domain of NF-κB (Jacobs & Harrison 1998). In addition, binding of IκBα to the p50/p65 complex provokes a profound conformational change in the p65 subunit inducing allosteric inhibition of NF-κB DNA binding (Huxford et al. 1998, Jacobs & Harrison 1998).

2.6.3 Regulation of the NF-κB activity

The biological action of the NF-κB complex is tightly regulated through its cytoplasmic retention by the association with IκB proteins. Different IκB proteins appear to target different combinations of NF-κB dimers (Baeuerle & Baltimore 1996). Various external stimuli (see Table 4), including cytokines, viral and bacterial products, pro-apoptotic, and necrotic stimuli, can all lead to the activation of NF-κB (Beg et al. 1993, Brown et al. 1993, Siebenlist et al. 1994,

Ghosh et al. 1998). Most of these activation signals target the inhibitory proteins, IκBs. The up-stream signaling pathways preceeding the IκB protein are divergent and may be specific for each inducer. These up-stream signaling pathways include PKC pathway (Shirakawa & Mizel 1989, Henkel et al. 1993), sphingomyelin pathway (Meichle et al. 1990), and reactive oxygen intermediates (Schreck et al. 1991, Manna et al. 1998).

Most of the known signaling cascades lead to activation of a large multi-protein complex called IκB kinase (IKK) (Brown et al. 1995, Chen et al. 1996, DiDonato et al. 1997, Mercurion et al. 1997, Regnier et al. 1997, Woronicz et al. 1997). The IKK-complex or its direct upstream activators may function as a critical collector / convergent point for activating signals coming from different signal transduction pathways and NF-κB activators. These signal transduction pathways are only partially known today. The catalytic subunits of IKK, IKKα (745 aa) and IKKβ (756 aa) are 52% homologous proteins and contain a N-terminal kinase domain followed by a leucine zipper region and C-terminal helix-loop-helix domain (Woronicz et al. 1997). They interact with each other, forming stable homo- or heterodimers. Rothwarf et al. (1998) reported that functional IKK is composed of equal amounts of IKKα and IKKβ and two IKKγ polypeptides. The major function of IKKγ is thought to link the IKK-complex to its up-stream activators.

The functional kinases of IKK, IKK-α and IKK-β (Zandi et al. 1997, Li et al. 1999), are responsible for the sequence-specific phosphorylation. Two essential regulatory subunits, IKK-γs, interact preferentially with IKKβ and are required for activation of the IKK complex (Rothwarf et al. 1998). IKK phosphorylates Ser-32 and Ser-36 of IκBα and Ser-19 and Ser-23 in IκBβ (Zandi et al. 1998, 1999). Phosphorylated IκBα is ubiquitinated at Lys-21 and Lys-22 (Palombella et al. 1994, Rodriguez et al. 1996), which targets it to degradation by 26S proteasome (Chen et al. 1995). Subsequently, the unbound NF-κB rapidly translocates to the nucleus and binds to its cognate DNA sequence, influencing transcription initiation. The proposed general activation pathway of NF-κB used by many inducers leading to nuclear translocation and biological action is presented in Appendix 1.

TABLE 4 Examples of NF-κB activating stimuli (Siebenlist et al. 1994, Baldwin 1996, Ghosh et al. 1998).

	NF-κB activating stimuli
Class	Examples
Cytokines	TNF-α, IL-1, IL-2, lymphotoxin
Mitogens	PMA, calcium ionophores, serum
Bacteria and their products	Shigella flexneri, Mycobacterium tuberculosis, bacterial LPS
Viruses and their products	HIV-1, CMV, EBV, HTLV-1, HBV, ds RNA
Environmental stress	UV light, ionizing radiation, oxidative stress
Chemical agents	Okadaic acid, ceramide, protein synthesis inhibitors

A few direct upstream activators of IKK are known. NIK (NF-κB inducing kinase) activates IKK by phosphorylating IKKα at Ser-176 (Malinin et al. 1997, Ling et al. 1998). It also phophorylates IKKβ (Woronicz et al. 1997). MEKK-1 can activate both IKK-α and IKKβ (Lee et al. 1998, Yin et al. 1998). In addition, casein kinase II (CkII) has been reported to phosphorylate Ser-32 of IκBα (Heilker et al. 1999) and two sites in the C-terminal end of IκBβ (Chu 1996). The 90-kD ribosomal S6 kinase also phosphorylates IκBα, promoting its degradation (Ghoda et al. 1997). Several other upstream activators of NF-κB have been identified. These include the Rho family of small GTPases (RhoA, CDC42, and Rac-1 (Perona et al. 1997), IRAK family members, IRAK-2, and MyD88, a death domain-containing adaptor protein (Muzio et al. 1997), and the TRF family members, TRAF-2, -5 and -6 (May & Ghosh 1998), TRAF1 (Carpentier & Beyaert 1999), TANK and TBK1 (Pomerantz & Baltimore 1999). However, their convergence point for the activation of the IKK is not yet known. Many accessory factors may also influence the activation of NF-κB, e.g. SUMO-1, which binds to IκBα and inhibits its degradation and thus NF-κB activation (Desterro et al. 1998).

In some situations, NF-κB has been reported to be activated without the IKK-complex and even without the degradation of IκB proteins. IκBα can be phosphorylated at Tyr-42 which leads to the detachment of the inhibitor from NF-κB (Imbert et al. 1996, Singh et al. 1996). However, this tyrosine-phophorylation does not lead to the degradation of inhibitor because it also inhibits the normal serine phosphorylation steps needed for degradation. The hypophosphorylated form of IκBβ, when complexed with NF-κB, can function as an activator of NF-κB (Suyang et al. 1996). In addition, various other phosphorylation steps are involved in the regulation of NF-κB activation. For example, PKA can phosphorylate the p65 subunit on Ser-276 (Zhong et al. 1997, 1998) and TNF-α on serine 529 (Wang & Baldwin 1998). Both of these modifications increase the transcriptional activity of NF-κB. IKK can also phosphorylate the p65 subunit on serine 536 in the transactivation domain (Sakurai et al. 1999). In addition, c-Rel (Mosialos et al. 1991) and p50 (Li et al. 1994) can be phosphorylated which increases their transcriptional activity. DNA-dependent protein kinase can phosphorylate both IκBα and IκBβ in their amino- and carboxyterminal regions, which is considered to regulate their DNA-binding properties in the nucleus (Liu et al. 1998). CBP (CREB-binding protein) and p300, which are both versatile co-activators, can interact with p65, when it has been phosphorylated on serine 276 by PKA (Zhong et al. 1998). This interaction increases NF-κB transcriptional activity by forming a molecular bridge between DNA-bound p65, other transcription factors, and the basal transcription apparatus (Gerritsen et al. 1997). NF-κB can also be activated by processing those NF-κB dimers which contain inhibitory precursor proteins (p105 and p100) and converting them into mature NF-κB proteins (Siebenlist et al. 1994, Verma et al. 1995).

2.6.4 NF-κB regulated gene expression

NF-κB is known to regulate the expression of more than 100 different genes, although in most cases, the specific target genes of various NF-κB dimers still remain to be identified. Various NF-κB / Rel / IκB transgenic and knockout mice have been created (reviewed by Baeuerle & Baltimore 1996, Ghosh et al. 1998, and Gerondakis et al. 1999) to study the physiological roles of these factors. Many of these gene alterations are accompanied by lethal or severe abnormalities in phenotype, emphasizing the importance of a functional NF-κB pathway in the maintenance of normal homeostasis.

TABLE 5 Examples of NF-κB –responsive genes (Siebenlist et al. 1994, Ghosh et al. 1998)

	NF-κB –responsive genes
Class	Examples
Cytokines and Growth factors	TNF-α, lymphotoxin, IL-1β, IL-2, IL-6, IL-8, interferon-β
Immunoreceptors	Immunoglobulin κ light chain, MHC-I, MHC-II
Adhesion molecules	Vascular cell adhesion molecule-1, endothelial-leucocyte adhesion molecule-1
Acute phase proteins	Angiotensinogen, complement factor B and C4
Viruses	HIV-1, HIV-2, CMV, SV-40, adenovirus
Transcription factors	p105, p100, c-rel, IκBα, c-myc, p53, p21

Many of the NF-κB target genes (Table 5) are involved in defensive cellular responses to stress, inflammation, and injury e.g. pro-IL-1β gene (Goto et al. 1999), murine manganese superoxide dismutase (Jones et al. 1997), iNOS (inducible nitric oxide synhtase) (Taylor et al. 1998), and the chemokine receptor, BLR1 (Wolf et al. 1998). Constitutively active NF-κB is a critical regulator and maintainer of B-cell maturation, e.g. expression of immunoglobulin kappa light chain (Sen & Baltimore 1986) and also required for T-cell immune responses (Attar et al. 1998). The p52 subunit has been reported to be required for normal spleen architecture and B cell-mediated immune responses (Caamano et al. 1998). NF-κB is involved in embryonal developmental processes e.g. c-Rel in normal limb bud development (Baeuerle & Baltimore 1996), Dorsal in dorsoventral axis patterning in Drosophila (Roth et al. 1989), and growth control by controlling the expression of several genes encoding growth factors and their receptors (Siebenlist et al. 1994). NF-κB can function either as an anti-apoptotic or a pro-apoptotic mediator, depending on the cell type (Baichwal & Baeuerle 1997). NF-κB activation has been shown to protect cells from several apoptotic stimuli (Beg et al. 1995, Beg & Baltimore 1996, Liu et al. 1996, van Antwerp et al. 1996, Mayo et al. 1997). NF-κB induced protection from apoptosis is thought to be linked to the avoidance of unnecessary cell suicide (Baichwal & Baeuerle 1997). NF-κB is also involved in cell transformation (e.g. Migliazza et al. 1994, Neri et al. 1996) and in metastasis of cancer cells to vascular endothelial cells (Tozawa et al. 1995).

Some of the members of the NF-κB/Rel gene family are autoregulated by NF-κB: p50/p105 (Ten et al. 1992), p52/p100 (Liptay et al. 1994), and c-rel (Grumont et al. 1993) contain functional κB-sites in their promoters. In addition, the inhibitor, IκBα, contains functional κB-sites in its promoter and, its expression is tightly controlled by NF-κB (Le Bail et al. 1993). These autoregulation loops help cells to maintain appropriate NF-κB and IκB levels inside the cell.

3 AIM OF THE STUDY

Aging is a genetically regulated process but its molecular control mechanisms are still unknown. However, both aging *in vivo* and *in vitro* involve an altered pattern of gene expression, which suggests that changes occur in transcription factor regulation during aging.

The specific aims of this study were:

1. To determine whether aging can affect the DNA-binding activities of ubiquitously expressed transcription factors, NF-κB, AP-1, and Sp-1 in various rat and mouse tissues *in vivo* and in replicatively senescent human fibroblasts *in vitro.*

2. To examine whether changes in NF-κB factor profiles are similar between aging *in vivo* and replicative senescence *in vitro.*

3. To characterize factors, which are involved in the age-related increase in constitutive nuclear NF-κB binding activity *in vivo.*

4. To elucidate the consequences of aging on the expression of NFκB-driven genes using replicative senescence of human fibroblasts as a model system.

4 SUMMARY OF THE MATERIALS AND METHODS

4.1 Animals and cells

NMRI-mice and Wistar rats, both sexes, were obtained from National Laboratory Animal Center, Kuopio, Finland. They consisted of two main age-categories: young mice which were 4 and old mice which were 24 months old, young rats which were 3-11 and old rats which were 26-30 months old. In addition, middle aged rats (18 mo) were used. The tissues studied were: liver, heart, brain, and kidney. The animals were killed using either CO_2 (rats) or cervical dislocation (mice). The tissues were removed, washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at -80 °C.

We also used two human lung fibroblast cell lines (WI-38 and IMR-90) obtained from the American Type Culture Collection. The cells were replicatively senesced by culturing (in DMEM supplemented with 10 % FCS and antibiotics, penicillin and streptomycin, Gibco-BRL). Passage number 20-24 represents young, actively proliferating cells and passage number 32-35 represents senescent cells. In addition, SV-40 transformed WI-38 cells were used as a control for active proliferation. The cultured cells were collected from plates either by lifting out in 1 ml of 1 x PBS using rubber policemen or by a standard trypsinization protocol, centrifuged (200 x g, 3 min), and stored at -80°C.

4.2 Reagents and kits

Oligonucleotides used for EMSA and supershift assays:
Consensus and mutated double-stranded NF-κB, consensus and mutated double-stranded Sp-1, and consensus and mutated double-stranded AP-1 were obtained from Promega and Santa Cruz companies.

The following antibodies were used for supershift assays, Western blot assays and immunocytochemistry:
Boehringer & Mannheim: anti-NF-κB antibody, p65 subunit
Cappel: HRP-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG, and rhodamine-conjugated goat anti-mouse IgG, rhodamine conjugated goat anti-mouse IgG
New England BioLabs: PhosphoPlus IκB-α (Ser 32) Antibody kit (cat. 9240)
PharMingen: p65 (14631A)
Rockland: anti-NF-κB/IκB kit (K-711) containing antibodies againts p50,p65, and IκBα
Santa Cruz: p50 (NL5, NLS, NLSX, H-119X), p52 (C-5, D-32, K-27,447X, K-27X), p65 (A, C-20, H-286X), c-Rel (C, CX, N, NX), RelB (c-19), c-fos (4), IκBα/MAD-3 (C-15), IκBβ (C-20), Bcl-3 (C-14), IKKα (H-744), IKKβ(H-470), IKKγ (FL-419), NIK (H-248)

4.3 Isolation of proteins for EMSA and Western blotting

Nuclear and cytoplasmic proteins were released and purified from samples according to the modified protocol described by Dignam et al. (1983). Frozen samples were thawn on an ice bath. Cytoplasmic proteins were released using hypotonic buffer. Supernatants containing cytoplasmic proteins, were collected by centrifugation, and stored in small aliquots at - 80 °C.

Pellets containing the nuclear proteins were suspended in low salt buffer and nuclear proteins were released by slowly increasing the KCl concentration of the sample to 0.4 M. Supernatants containing the soluble nuclear proteins, were collected by centrifugation and stored in small aliquots at -80 °C. The total protein concentration of samples was measured using Protein Assay Reagent (Bio-Rad) and ELISA-method according to their protocol.

4.4 EMSA (Electrophoretic mobility shift assay)

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 manufacturer.

Nuclear protein samples (10 µg) and cytoplasmic protein samples (25 µg) were incubated (15 min, room temperature) with labeled probe (20 000 cpm). The salt concentration was balanced by filling all samples to the same volume with the low salt/high salt (2:1) buffer used in protein isolation. Unspecific binding was blocked using poly dI/dC (Pharmacia). When analyzing the NF-κB binding, 10% Nonidet P40 (BDH) was added. The inactive cytoplasmic NF-κB complex was activated using 2% (w/v) deoxycholic acid (Sigma). The reaction was stopped using DNA loading buffer. The bound and unbound probes were

separated in native polyacrylamide gel electrophoresis (PAGE), the gel was dried on a Whatmann paper (3MM) (80 °C, 1 h), and the results were visualized on an autoradiography film (NEN, Fuji) (1-2 d, at -80 °C) or on Storm PhosphoImager (Molecular Dynamics).

4.5 Supershift assay

The protein components in the specific NF-κB complex were identified using the supershift assay. After 10 min binding reaction (described in 4.4), a specific primary antibody against different components of the NF-κB complex was added to the sample, incubated further (30 min, 4 °C), and a normal gel retardation assay and visualization were performed.

4.6 Western blot assays

Nuclear (15 µg) and cytoplasmic proteins (20µg) were denatured by heating in buffer containing SDS. The prestained molecular weight standard mixture (low range) (BioRAD) was similarly denaturated. Proteins were separated either in 10% or 12% SDS-PAGE gel electrophoresis and transferred from gel to a PVDF-(Hybond) or Immobilon-P (Millipore) membrane using a semi-dry transfer technic (Pharmacia LKB Multiphor II).
Membranes were blocked prior to immunostaining with 5% fatfree milk powder in PBS, 0.1% Tween (1 h at room temperature or over night at 4 °C). After that, membranes were incubated with a primary antibody for 2 h at room temperature. After washing, bound antibodies were detected on autoradiography film (NEN, Fuji) with either goat anti-rabbit HRP or rabbit anti-mouse HRP and ECL-Western Blot Chemiluminescence Reagents according to the protocol of manufacturers (Amersham, Du Pont, or Pierce).

4.7 UVB-irradiation

WI-38 fibroblasts were cultured to 70 - 80% confluence. Before the UV-illumination, normal growth medium was aspirated, cells were washed once with PBS and DMEM (w/o phenol red and serum) was added on each plate. The cells were UV-illuminated with UVB-light (Philips, TL 20W/12) giving the energy of 153 mJ/cm^2 without any lid at room temperature. After that, the medium was aspirated and normal growth medium was added to each plate. Cells were collected from plates as described in 4.1 after 0 h, 6, 12, and 24 hrs incubation (37 °C, 10% CO_2). Controls were placed under the same conditions without UVB-irradiation.

4.8 Transfections

Transient co-transfections were made either with the calcium phosphate precipitation method (Sambrook et al. 1989) or with the lipofection method (DOTAP, Boehringer & Mannheim). The plasmids used were various kind of NF-κB-enhancer-driven CAT reporter genes together with the lacZ reporter gene (pCH110, Amersham), which was used as a control for transfection efficiencies and translation capacities. The expression of CAT was analyzed with a CAT-ELISA kit (Boehringer) and that of β-galactosidase by using CPRG substrate (Boehringer). For transfection studies, the p50-CAT3 construct was established by PCR cloning. This carries the functionally active κB-site of p105 gene (position -76 - -55 bp) cloned in Kpn I-site (-5) of pCAT3-promoter vector (Promega).

4.9 Northern hybridization

Total RNA was isolated from rat livers using TRIzol (GibcoBRL) reagent and the protocol of the manufacturer. The poly(A)-mRNA was further purified with PolyATract (Promega). PCR primers used were designed with Primer Detective 1-01 software (Clontech). Gene specific fragments for riboprobes were generated by PCR and cloned into pGEM-T Easy vector (Promega) and verified by sequencing. The ^{32}P-labelled riboprobes used in hybridizations were generated with Strip-EZ kit (Ambion).

300 ng of rat liver mRNA were separated in denaturing agarose gel electrophoresis (3.3 V/cm voltage gradient, 2.5 h), transferred to nailon membrane (Magna Charge, MSI) by a downward capillary process and fixed by UV crosslinking (72 mJ/cm^2) (Stratalinker 1800, Stratagene).

Hybridization was performed in modified ''high-stringency'' Church buffer (Church & Gilbert 1984) at 55-60 ºC depending on the probe length and GC content. Subsequently, the filters were washed (1 x SSC, 0.2% SDS, 68 ºC, 30 min and 0.1 x SSC, 0.2% SDS 68 ºC, 1h) and signals were visualized on Storm 860 PhosphoImager (Molecular Dynamics) after 1-5 days of exposure. After stripping, the membranes were reprobed with the cyclophilin riboprobe, which was used as an internal standard.

4.10 Immunocytochemistry

Cultured fibroblasts were first fixed with 100% methanol (5 min, -20 °C). Then unspecific binding was blocked by incubating in 3% BSA, 2% saponine in PBS (1 h, room temperature). Then, the samples were incubated with a primary

antibody (anti-NF-κB/IκB kit, K-711, Rockland) (1 h, room temperature) followed by incubation with secondary antibody (rhodamine conjugated goat anti-mouse IgG, Cappel) (1 h, room temperature). Finally, the cells were incubated with nuclear dye (Hoechst 33258, Sigma) and embedded.

4.11 Histochemistry

A stained liver sample from each of the animal used was studied under the microscope to check that tissue histology was normal. The liver samples were frozen in iso-pentane placed in a liquid nitrogen bath and stored at – 80°C. Tissue samples were sectioned (10 μm thick) and allowed to stand at room temperature for 5 – 10 minutes. Then, they were stained with Harris' hematoxyline and eosine. Briefly, samples were first stained with Harris' hematoxyline (10 min), washed briefly under running water, dipped twice in 0.25% HCl, stained with eosine (3 min) and dehydrated (96% EtOH, 2 min and twice at 100% EtOH, 2 min each) and finally cleared with xylene (5 min) and embedded.

4.12 Statistics

The autoradiographs from EMSA were analyzed by using Image Grapper 2.1 (Neotech Ltd.) and Image 1.43 programs to measure the mean density of binding bands. In addition, Northern hybridization and part of the EMSA results were visualized on Storm 860 PhosphoImager (Molecular Dynamics). Results were statistically analyzed with univariate analysis of variance and non-paired Student's t-test.

5 REVIEW OF THE RESULTS

5.1 Aging induced up-regulation of constitutive, nuclear binding activity of NF-κB in tissues

Aging involves changes in gene expression reflecting age-related changes in gene regulation. Key components in the gene regulatory machinery are inducible transcription factors, but little is known about the possible aging-associated changes in their regulation. We started to study age-related changes in the binding activity of three ubiquitously expressed transcription factors (NF-κB, AP-1, and Sp-1) using an EMSA method. Our results revealed age-related changes in their binding activity.

Aging induced a significant and consistent increase in constitutive, nuclear NF-κB binding activity in all tissues studied (heart, liver, brain, and kidney) in both species used (mice and rats) (I-III, IV). This effect appeared both in males and in females. The level of increase depended on which tissues were examined. The increase was most prominent in heart (123%) and liver (101%). In brain, the increase was most prominent in cerebellum and in frontal cortex. On the contrary, age-related changes did not occur in hippocampus. The up-regulation of nuclear NF-κB binding activity was already observable in middle-aged rats (II, III). Supershift assays were used to evaluate the protein components of the nuclear NF-κB dimer. They showed that the nuclear NF-κB complex contained p50, p52, and p65 proteins (I). However, the age-related increase in NF-κB binding activity was specific for the nuclear fractions, because no statistically significant, age-related changes were detected in DOC-induced, cytoplasmic NF-κB binding activity in any of the tissues studied (I-III, IV).

Aging strongly down-regulated the binding activity of nuclear Sp-1 in mouse heart and brain and in rat liver and heart (I, III). In contrast, no age-related changes were detected in rat brain (III). The binding activity of AP-1 showed a high inter-individual variance in most of the tissues studied (I-III). The greatest significant age-related decreased in AP-1 binding activity was detected in mouse heart (-28%). No statistically significant sex-related differences were found in the responses to NF-κB, Sp-1, and AP-1.

The histochemical stainings of samples showed that there were no tumours or inflammational changes in tissues, which could have induced the observed changes in NF-κB binding activity.

5.2 Effect of aging on the protein levels of NF-κB subunits and their main inhibitors

The increased nuclear NF-κB binding activity could be due to the age-related changes in its protein components. Thus, we studied the protein levels of various NF-κB subunits using Western blotting. Our results showed that aging involves changes in some NF-κB subunits, but these changes were tissue-specific.

In the aging heart, the level of nuclear p52 protein component (I, III) and in the aging liver, the levels of both nuclear and cytosolic p52 and p65 protein components of the NF-κB complex increased strongly (III, IV). However, in rat brain, no age-related changes were detected in the protein components of NF-κB studied (p50, p52 or p65) (II). Furthermore, the level of p50 protein did not show age-related changes in any of the tissues studied, either in the cytosolic or nuclear fractions. The nuclear level of c-rel protein showed a high inter-individual variance with age and the cytosolic level of c-rel was unaffected by age in rat liver (IV). In addition, Western blots of p52 and p65 subunits showed a slower migrating band in all samples, which could indicate the presence of post-translationally modified forms of these proteins.

Age-related changes in the phosphorylation of NF-κB subunits may influence their binding activity (Li et al. 1994, Zhong et al. 1998) and dephosphorylation experiments were carried out to identify these possible changes. However, these experiments did not, reveal any changes in phosphorylation levels of p65 and p52 subunits in aged liver samples when they were compared to the liver samples from young animals (IV).

The age-related increase in nuclear NF-κB binding activity could also be due to aging-associated changes in the protein levels of its inhibitors, which were also studied. Surprisingly, aging did not affect the protein levels of most NF-κB inhibitors. The protein levels of IκBα and Bcl-3 did not show any age-related changes either in nuclear or in cytosolic fractions in any of the tissues studied (I-III, IV). The level of cytosolic IκBβ was also unaffected by age. However, the level of nuclear IκBβ decreased with age in rat liver. (IV)

5.3 Replicative senescence induced changes in NF-κB

Replicative senescence of human fibroblasts (WI-38 and IMR-90) induced, on the contrary, a strong decrease in nuclear NF-κB binding activity (III, V). A similar decrease in nuclear DNA binding activity was also seen with Sp-1 (III,

V) and AP-1 factors (V) in replicatively senescent fibroblasts. However, the nuclear Sp-1 binding activity was strongly up-regulated by SV-40 transformation (III). Supershift EMSA assays showed that the specific NF-κB complex contained p50 and p65 components but not those of p52 and c-Rel in cultured human fibroblasts (V).

In addition, replicative senescence induced other kinds of changes in the protein levels of NF-κB components, which differ from aging *in vivo*. In replicatively senescent human fibroblasts, protein levels of p50 and p52 components of NF-κB complex were decreased in nuclear fractions but not in cytosolic fractions (III). On the other hand, the cytosolic level of p65 protein was increased. The levels of the inhibitory proteins studied, IκBα and Bcl-3, also showed a slight decrease in senescent fibroblasts when compared to both early passage and SV-40 transformed fibroblasts (III). However, replicative senescence of human fibroblasts did not affect the protein levels of cytoplasmic IKKα and IKKβ (V) involved in activation of NF-κB by many inducers.

5.4 Effect of aging on the protein kinases controlling the activity of NF-κB

Generally, activation of NF-κB involves the IKK complex, which specifically phosphorylates IκB proteins, triggering their ubiquitination and degradation. Then the unbound NF-κB dimer can translocate to the nucleus and bind to cognate DNA. We studied protein levels of the IKK protein components to determine if there were any possible age-related changes in their concentrations. Our results showed that aging did not affect the protein levels of the kinases forming the functional IKK complex (IKKα and IKKβ) or its regulatory subunits (IKKγs) in rat liver (IV). However, IKKβ was mainly located in nuclear fractions and was almost totally absent in cytoplasm both in young and in aged samples. Surprisingly, aging did not affect the level of the Ser-32 phosphorylated form of IκBα (IV), which is known to be involved in NF-κB activation by many inducers (Henkel et al. 1993). The protein level of NIK, kinase activating IKK complex, was also unaffected by age in rat liver (IV).

5.5 Expression of NF-κB and IκB genes in rat liver

The age-related increase in the levels of some NF-κB proteins observed in this study may be due to their increased expression with age. In addition, the expression of many NF-κB and IκBα inhibitor genes are autoregulated by NF-κB. Therefore, the expression levels of NF-κB mRNAs and their main inhibitors were studied. Northern hybridization experiments showed that mRNA levels of p50, p52, and p65 components were slightly increased with age, but there

were no statistically significant age-related changes in the mRNA levels of NF-κB members studied (p50, p52, p65 and c-rel) (IV). The mRNA levels of the main inhibitors (IκBα and -β) also did not show any significant changes with age (IV).

5.6 Attenuation of NF-κB signalling response during cellular senescence

We studied the activation mechanism of the NF-κB pathway during the defensive response by irradiating cultured fibroblasts with UVB light, which is both a potent NF-κB activator and a common inducer of environmental stress. The exposure of early passage fibroblasts to UVB light blocked their proliferation and induced morphological changes such as a flat phenotype (V) which has previously been reported to occur during replicatively induced cellular senescence (Cristofalo & Pignolo 1993). However, a caspase-3 activity assay showed that the UVB dose used did not induce apoptosis in these fibroblasts (V).

Replicative senescence of human WI-38 and IMR-90 fibroblasts induced a prominent attenuation in the NF-κB-mediated signaling in response to UVB-light (V). This attenuation was most prominent in DNA binding activities of nuclear NF-κB complex and in CAT reporter gene assays. Similar senescence-related attenuation to the UVB light response was also observed in the DNA binding activities of nuclear AP-1 and Sp-1 (V). Supershift assays showed that the specific NF-κB binding complex contained p50 and p65 components but not those of p52 and c-rel (V).

UVB-induced NF-κB activation involves the IKK-complex and degradation of IκBα. The cytosolic protein levels of both IKKα and IKKβ decreased after an activating UVB signal, especially in replicatively senescent fibroblasts (V). This was accompanied by a prominent increase in the phosphorylation level of IκBα at Ser32, which was clearly stronger in early passage fibroblasts. UVB exposure clearly reduced the cytosolic protein level of IκBα both in early and late passage fibroblasts as well as SV-40 transformed WI-38 fibroblasts 4 and 12 hours after UVB exposure (V). The level of nuclear IκBα was also reduced after UVB induction in all samples. The reduction of the cytosolic IκBα protein level was accompanied by the nuclear translocation of p65 subunit seen in immunocytochemical stainings (V). Western blot assays also verified that the nuclear levels of p52 and p65 proteins but not those of p50 and c-rel increased slightly after UVB light exposure (V). SV-40 transformation of fibroblasts increased the level of both cytosolic and nuclear IκBα compared to those of early and late passage fibroblasts (V).

Transient transfection assays showed that the response to UVB exposure in NF-κB complexes represents the transcriptional activation of the NF-κB signaling pathway, although the response was clearly attenuated in senescent fibroblasts (V). Our experiments with protein kinase inhibitors (tyrphostin AG

126 and herbimycin A), and phosphatase-inhibitors (okadaic acid and sodium fluoride) further showed that UVB-induced increase in NF-κB signaling is under the regulation of protein phosphorylation. Furthermore, UVB induced a NF-κB activation signaling cascade including most probably the MAPK/SAPK pathway, because the kinase inhibitors used block this pathway specifically (Cox et al. 1996, Takahashi & Berk 1996) and we observed that both of these kinase inhibitors were also able to block the activation of NF-κB signaling after UVB-exposure (V).

5.7 Down-regulation of NF-κB promoter activity with replicatively induced cellular senescence

To study the possible effects of the observed age-related changes in the NF-κB signaling pathway to the expression of its target genes, transient transfection experiments were carried out in cultured human fibroblasts. In senescent WI-38 fibroblasts, a significant down-regulation of NF-κB promoter activity with senescence was seen in all transient transfection assays made using diverse NF-κB-enhancer-driven CAT reporter genes (III, V). UVB-exposure of fibroblasts significantly activated the expression of the NF-κB-enhancer-driven reporter genes both in early and in late passage fibroblasts. However, the expression of NF-κB-driven CAT reporter genes in response to the UVB activation signal was significantly stronger in early passage than in late passage fibroblasts in all constructs used (III, V). The TK-5 construct carrying six κB-sites of TNF-α promoter in its enhancer was more effective than the TK-10 construct with two κB-sites of the TNF-α promoter (V). The same down-regulatory response in target gene expression with replicative senescence was also seen in a p50-CAT-reporter gene construct carrying the functionally active κB-site of p105 subunit of NF-κB (Fig. 1). p50-CAT3 values have not been published before.

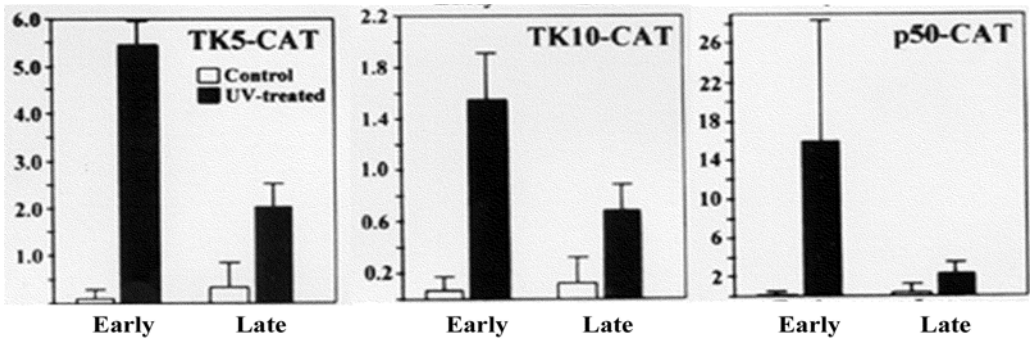

FIGURE 1 UVB induced expression of CAT-reporter genes in early and late passage WI-38 fibroblasts transiently co-transfected with TK5-CAT,TK10-CAT, or p50-CAT3 plasmids together with pCH110 control plasmid. (Amersham). The activity of CAT was calculated per β-galactosidase expression (pg CAT/mU β-gal.). Values are mean +-SD (n=3 in each group, $p < 0.01$).

6 DISCUSSION

Aging is considered to be a genetically programmed event in this study, like early development, both of which involve changes in gene transcription. The molecular control mechanisms of aging are still largely unknown, even though there have been many different theoretical approaches used to study aging mechanisms (reviewed e.g. by Dice 1993). However, no single theory has gained unequivocal acceptance. Control mechanisms and causes of aging are thought to be multifactorial. The expression of many genes changes during aging both *in vivo* and *in vitro*, suggesting that age-related changes occur in their regulation. Transcription factors are the key regulators in gene expression. Inducible transcription factors may, thus, also be involved in orchestrating age-related changes in gene expression.

The NF-κB family of transcription factors might be an ideal regulator of age-related changes in gene expression. NF-κB is ubiquitously expressed, it is inducible and its individual members perform specific and distinct tasks and have also cell-type- and tissue-specific functions. In eukaryotic cells, NF-κB coordinately controls the expression of many genes, which have significant and diverse biological functions. In addition, the activation of NF-κB does not require protein synthesis, which is known to be reduced with age.

6.1 Up-regulation of constitutive, nuclear NF-κB binding activity in tissues

In this study, a significant and consistent increase with age was seen in constitutive, nuclear NF-κB binding activity in all tissues, in both sexes, and in both species studied. The results suggest that this increase is a general age-related change in tissues and, hence, a regulated process. Aging causes mostly deleterious changes in cells and down-regulation of cellular functions (Masoro 1995, Richardson & Holbrook 1996, Wheaton et al. 1996). Few constitutive age-related increases have been reported to occur in transcription factor binding activity (e.g. Dimri & Campisi 1994, Walter & Sierra 1998). In rare cases, their

binding activity has been reported to be constitutively decreased with age, as in the case of AP-1 (Riabowol et al. 1992) and Sp-1 (Ammendola et al. 1992).

The level of increase in NF-κB binding was subject to extensive variation from 40% in brain to 123% in heart, which may reflect tissue-specific changes in the regulation of NF-κB signaling during aging. There are probably many molecular and functional reasons for this increase and it may also vary in different cell-types and tissues. Supershift assays showed that the bound NF-κB complexes contained p50, p52, and p65 proteins.

The observed increase in their binding activity may partly be due to the post-translational modifications of NF-κB subunits. However, modifications of NF-κB do not usually lead to its nuclear translocation. Translocation requires the removal of IκB proteins. Thus, mechanisms, which control activation signals and nuclear translocation of NF-κB proteins, are also suggested to be involved in this up-regulation.

6.2 Activation of NF-κB complex

The activation of the NF-κB complex is tightly regulated through its cytoplasmic retention in association with IκB proteins (Siebenlist et al. 1994, Ghosh et al. 1998). Inducers of NF-κB generally target IκB proteins, although some exceptions are known. Examples of these exceptions are UVC induction in some cell types (Li & Karin 1998), treatment of cells with tyrosine phosphatase inhibitors and during reoxygenation of hypoxic cells (Impert et al. 1996, Singh et al. 1996). NF-κB is activated using signal pathways which do not include the IKK-complex or degradation of IκB proteins.

6.2.1 Transient activation of NF-κB

We studied the activation of NF-κB by transiently activating it with UVB-light, which is an effective inducer of NF-κB signaling (Devary et al. 1993). Our results showed that UVB induced activation of NF-κB was clearly attenuated in replicatively senesced human fibroblasts. Supershift assays showed that bound complexes contained p50 and p65 proteins after UVB-induction but neither p52 nor c-Rel were present. Western blot assays of NF-κB proteins, however, did not show any significant senescence–related changes.

Replicatively senescent cells have a generally reduced ability to respond to external signals (Richardson & Holbrook 1996). Phosphorylation of an IκB protein is one of the key regulation steps in NF-κB activation after UVB-induction. We observed a clear decrease in the level of Ser-32 phosphorylation in IκBα in senesced fibroblasts after UVB induction. This was accompanied by disappearance of IκBα protein in the later phase of activation and concomitant nuclear translocation of p65 protein. These results suggest that attenuated NF-

κB activation with replicatively senescence is due to depressed signal induced phosphorylation of IκBα inhibitor.

IKK-complex phosphorylates IκBα specifically at Ser-32 and Ser–36 (Traenckner et al. 1995). The protein levels of catalytic IKK kinases (IKKα and -β) were not affected by replicative senescence, but their catalytic activity might have been reduced and this could account for the observed attenuation. In addition, senescence-related changes in regulatory subunits of the IKK complex (IKKγ1 and -γ2) may be involved. However, their possible roles in this attenuation require further study. The attenuated activation of NF-κB in senesced cells may also be due to age-related changes in up-stream signaling cascades, which activate IKK kinases. Both oxidative damage to membranes and DNA alterations in the nucleus have been reported to be involved in the activation of NF-κB by UV (Piette et al. 1997, Legrand-Poels et al. 1998). The UVB-activation pathway starts from TNF receptor 1, which rapidly associates with TRAF-2, in keratinocytes (Tobin et al. 1998). Several subsequent studies showed that UVB radiation induces the Ras-related signaling pathway and activates mainly the JNK (in SAPK) pathway (Engelberg et al. 1994, Devary et al. 1992). MEKK-1 directly activates the IKK-complex *in vitro* and induces the site-specific phosphorylation of IκBα *in vivo* (Lee et al. 1998).

The experiments with specific kinase inhibitors of the MAPK/SAPK pathway showed that UVB induced NF-κB activation is dependent on phosphorylation and the activation pathway most probably include kinases of the MAPK/SAPK pathway. Activation of the MAPK cascade has been shown to be reduced in cellular senescence (Liu et al. 1996, Whisler et al. 1996), which may be the reason for the attenuated NF-κB responses in senescent cells observed in this study. However, the experiments with phosphatase inhibitors suggest that protein phosphatases are not involved in this attenuation. The possible up-stream signaling component affected by senescence may be common to NF-κB and AP-1 pathways, and perhaps also common with the Sp-1 pathway, because replicative senescence attenuated all of their binding after UVB-induction. A similar age-related decrease in the induction of both NF-κB and AP-1 binding activity has also been reported to occur in old rat spleen lymphocytes after concanavaline A (con A) activation (Pahlavani et al. 1996). One candidate kinase, which could be affected, is MEKK-1, which is a critical coordinate regulator of both NF-κB and AP-1 stress response pathways (Lee et al. 1998).

In vivo, a similar decrease in NF-κB binding activity with age has been reported to occur in lymphocytes, e.g. in human T-cells (Kilpinen et al. 1996, Trebilcock & Ponnappan 1996), in mice helper T cells (Albright et al. 1995) as well as in rat spleen lymphocytes after con A induction (Pahlavani et al. 1996). However, Trebilcock & Ponnappan (1996) reported that phosphorylation and ubiquitination of IκBα were not altered with age in TNF-α treated human T cell. These findings point to a general down-regulation of transient NF-κB activation pathway with age, at least in lymphocytes and in cultured fibroblasts. Up-stream signalling pathways and activation mechanisms affected by age may vary in different cell types and tissues. Further studies are needed to identify

the senescence affected molecule(s) in the NF-κB activation pathway and possible tissue-specific variation in signaling.

6.2.2 Constitutive activation of NF-κB binding activity in aged tissues

The age-related increase in nuclear NF-κB binding activity was constitutive in tissues. Tissue supershift assays demonstrated that the DNA-bound NF-κB complex contains p50, p52, and p65 proteins. Western blot assays further showed that the level of p52 and p65 proteins was clearly increased in aged livers and nuclear p52 was elevated in aged hearts. On the contrary, the level of p50 protein was unaffected by age. No age-related changes in NF-κB proteins were detected in aged brains. These results emphasize the tissue-specific variation in NF-κB complex and in the regulation of NF-κB signaling during aging.

In aged heart and liver, a part of the observed increase in NF-κB binding activity is most probably attributable to the increased protein level or post-translational modifications of p52 and p65 components. Northern hybridization assays, however, showed that the increased protein levels of p52 and p65 were not due to their increased expression. The expression of other NF-κB genes (p105 and c-rel) and their main inhibitors (iκBα and -β) were also unaffected by age. The observation that only nuclear NF-κB showed increased binding activity with age suggests that processes which control NF-κB nuclear translocation, its half-life in nucleus or binding activity are involved. We studied the protein levels of kinases (NIK and the components of functional IKK-complex) involved in activation of NF-κB by many inducers, but we did not observe any aging-associated change in these proteins. We did not observe any age-related change in the protein levels of the main cytoplasmic NF-κB inhibitors (IκBα and IκBβ), degradation of which is required for NF-κB activation by many inducers. The level of Bcl-3 inhibitor was also unaffected by age in all of the tissues studied. The level of Ser-32 phosphorylated IκBα was also unaffected in aged rat livers. These findings suggest that the increase in NF-κB binding activity in aged tissues does not involve degradation of these IκB proteins and furthermore that the IKK-complex is not involved. One mechanism to explain the up-regulating NF-κB binding activity during aging could involve post-translational modifications of NF-κB proteins, stabilizing the complex and increasing its binding activity to DNA. We observed additional, higher molecular weight bands in the Western assays of p52 and p65 subunits in liver, which supports this modification hypothesis. In addition, accessory factors, which are able to facilitate NF-κB binding to DNA, may also be involved.

There are many possible ways that NF-κB proteins can be modified by e.g. phosphorylatation, ubiquitination, and acetylation. Dephosphorylation experiments, however, showed that the phosphorylation level of p52 and p65 proteins was not changed in aged livers. In other tissues, such as brain, where increased binding activity with age was not accompanied with increased level of NF-κB proteins, the increased nuclear binding activity may be due to this

kind of modification of NF-κB proteins, which can induce changes in their cellular location and binding activity (Mosialos et al. 1991, Li et al. 1994, Naumann & Scheidereit 1994, Zhong et al. 1998). Especially in these tissues, the increased nuclear NF-κB binding activity may also partly be attributable to the presence of an accessory protein(s),such as HMG1(Y) (Zhang & Verdine 1999), which might be affected by age and which could enhance NF-κB binding to DNA. The possible involvement of these modifications needs to be studied.

Part of the increased nuclear level of p52 subunit in aged hearts and livers may also be due to the increased signal induced 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 (Betts & Nabel 1996). Up-stream signaling cascades leading selectively to the degradation of p100 could be affected by age. However, these signaling pathways are still largely unknown.

Currently, it is not known, how induction leads to the phosphorylation of a specific IκB protein, which can then further activate a particular NF-κB dimer. The only observed age-related change in IκB proteins was a decreased protein level of nuclear IκBβ in aged livers. This decrease suggests that either the nuclear translocation of IκBβ decreases with age or its degradation is enhanced in the nuclei of aged cells. Both of these processes are regulated, but the mechanism is still largely unknown. However, nuclear IκBβ can dissociate NF-κB from DNA (Zabel & Baeuerle 1990) terminating the expression of a target gene. The decreased nuclear level of IκBβ in aged cells may not be able to do this as effectively as it can in young cells. This reduction in nuclear IκBβ protein level may play a part in the increased nuclear binding activity of NF-κB in aged livers.

The observed increase in NF-κB binding activity occurred only in nuclear fractions. Therefore, it is also possible that in aging tissue, a kinase (kinases) is activated which is capable of phosphorylating IκB protein (in other residue(s) than the studied Ser-32 of IκBα) permitting the release of NF-κB without triggering the degradation of the inhibitor. One such kind of kinase might be that which can induce Tyr-42 phosphorylation of IκBα (Imbert et al. 1996). In addition, DNA-PKcs (DNA-dependent protein kinase catalytic subunit) can phosphorylate both IκBα and IκBβ in its N- and C-terminal ends (Liu et al. 1998). DNA-PKcs phosphorylation of IκBα increases its binding to NF-κB and removal of DNA-PKcs activity induces constitutive nuclear NF-κB binding activity with the increased level of nuclear p65 protein. We have previously shown that DNA-PK activity decreases with replicative senescence (Salminen et al. 1997). Thus, the possible decrease in DNA-PK activity in aging tissues could induce the observed increase in nuclear NF-κB binding activity and also increase the level of p65 subunit as observed here in aged livers. The possible involvement of this (these) kinase(s) remains to be determined. Shelton and coworkers (1998) showed in fibroblasts that the senescence state mimics a strong inflammation-type of response, which may be involved in the activation of NF-κB binding in these cells. Thus, further studies are needed to identify the

activation mechanism by which aging increases the nuclear NF-κB binding activity and protein levels of only some NF-κB subunits in animal tissues.

6.3 NF-κB driven transcription

Our results from cultured, replicatively senescent fibroblasts suggest that age-related changes in NF-κB factor are repressive to target gene expression. All transfection studies using different kinds of -κB enhancer constructs carrying reporter vectors showed clear decreases in the level of reporter gene expression with replicative senescence. However, the NF-κB binding activity also decreased in replicatively senescent fibroblasts. This could induce the observed decrease in the expression of NF-κB-driven target genes. *In vivo*, a similar down-regulatory effect of NF-κB on gene expression with age has been reported with the rat androgen receptor gene (Suparkar et al. 1995). NF-κB is also a key regulator of the induction of many immunoregulatory genes, including IL-2 and IL-2 receptor alpha (Hoyos et al. 1989, Pimental-Muinos et al. 1994) expression of which is reported to be significantly suppressed during aging in conjunction with decreased NF-κB activation (Ponnappan et al. 1998, 1999). The age-related decrease in NF-κB activation has been proposed to be involved in immune senescence (e.g. Ponnappan 1998). These examples suggest that down-regulation of NF-κB target genes also occurs in aged tissues.

Northern hybridizations of NF-κB and their main inhibitors, some of which are autoregulated, showed that their expression was not affected by age in rat liver. These results further suggest that aging-associated changes in NF-κB signaling are not able to enhance expression of target genes. For example, the expression of p100/p52 gene, a precursor of p52 protein, is upregulated by p65 (p65/p50) and repressed by p52 protein (Lombardi et al. 1995). The p65 protein has also been proposed to play a critical role in the control of IκBα expression (Beg et al. 1995). The levels of neither p100/p52 nor IκBα mRNAs were, however, significantly affected by age in rat liver. In human monocytes, the up-regulation of p100/p52 expression was reported to be dependent mainly on the tyrosine kinase pathway (de Wit et al. 1998), which appears to be unaffected by age.

Different NF-κB dimers bind with variable affinity to a κB-site (Perkins et al. 1992, Hansen et al. 1994, Pan & McEver 1995, Grigoriadis et al. 1996). The influence of aging-associated changes on NF-κB signaling to the expression of target genes is, thus, gene specific and mainly depends on the structure of their κB-sites. The observed changes in the NF-κB factor in different aged tissues most probably leads to gene- and tissue-specific alterations in the expression of its target genes. For example in aged livers, the compositions of nuclear NF-κB dimers may change during aging because of the increasing level of p52 and p65 proteins. These changes may further target the increased NF-κB binding activity selectively to the expression of those target genes having high binding affinity

for the NF-κB complexes containing p52 and p65 subunits. Observed age-related increase in p52 subunit in heart and liver may, for example, affect humoral immunological responses in these tissues because mice deficient in p52 subunit was considered to indicate the involvement of p52 protein in humoral responses (Caamano et al. 1997). The specific target genes for different NF-κB dimers are, however, largely unknown at this moment making it difficult to specify the possible aging-associated alterations in their expression *in vivo*.

NF-κB interacts with many other transcription factors and accessory factors in the promoters of target genes (e.g. Perkins et al. 1993, Sanceau et al. 1995, Xia et al. 1997, Dechend et al. 1999). Thus, the increased level of promoter bound NF-κB alone may be insufficient to induce target gene expression if there is no concurrent increase in other bound transcription factors on these promoters. The aging-associated increase in nuclear NF-κB binding activity may partly be due to the age-related changes in accessory factors, such as HMG1(Y) (Zhang & Verdine 1999). These could facilitate the binding of NF-κB to cognate DNA, at least in some tissues, such as brain, where protein levels of NF-κB subunits were unaffected by age.

However, the number of studied target genes is very limited and these results do not exclude the possibility that age-related changes in NF-κB signaling may also enhance the expression of some genes *in vivo*. These genes may be involved in the onset of some age-related changes in cellular function. One of the NF-κB target genes, c-myc, is a proto-oncogene and is progressively upregulated during aging in liver, heart, brains, and skin (Semsei et al. 1989). However, nothing is known about the involvement of NF-κB in the up-regulation of this gene during aging. On the contrary, the expression of inducible nitric oxide synthase gene (iNOS) (Bradley et al. 1998), another NF-κB target gene, is increasing during aging (e.g. Vernet et al. 1998) and this increase is associated with enhanced NF-κB activation in vascular smooth muscle cells (Yan et al. 1999). Furthermore, McCann and co-workers have published the nitric oxide hypothesis of aging (McCann et al. 1998) in which they propose that recurrent infections, which induse iNOS to produce NO (nitric oxide), play a significant role in producing aging changes in tissues via release of toxic quantities of NO. This way, enhanced NF-κB activation during aging might be associated with aging-associated changes in tissues. In addition, NF-κB regulates the expression of many antiapoptotic genes, for example c-IAP2 (inhibitor of apoptosis) (Chu et al. 1997) and cyclo-oxygenase-2 (von Knethen et al. 1999) which may link the increased nuclear NF-κB binding activity with age to the elevated apoptosis resistance of aged cells.

6.4 Comparison of aging *in vivo* and replicative senescence *in vitro*

Our results obtained from *in vivo* and *in vitro* studies of aging are partly conflicting. The increased nuclear NF-κB binding activity with age together

with tissue-specific increase of some NF-κB proteins could not be verified in our *in vitro* model of cellular senescence. The decreased NF-κB activation of senescent fibroblasts observed here was, however, parallel to decreased NF-κB activation reported to occur in lymphocytes *in vivo* (Pahlavani et al. 1996, Trebilcock & Ponnappan 1996) suggesting that the transient NF-κB activation pathway functions similarly in aging either *in vivo* or *in vitro*, at least in these cell types. The observed down-regulation of NF-κB binding activity in replicatively senescent fibroblasts may also decrease the expression of NF-κB target genes as observed here. The results obtained from *in vivo* studies of NF-κB in lymphocytes suggest, however, that some of NF-κB target genes, such as IL-2 receptor alpha (Trebilcock & Ponnappan 1996), are also down-regulated in aged cells *in vivo*. However, the down-regulatory effect of NF-κB on target gene expression may be highly selective, being different in other cell and tissue types from cultured fibroblasts and lymphocytes.

The observed differences in NF-κB signaling *in vivo* versus *in vitro* may arise, at least partially, due to the difference in cell types and populations in the tissues and in cultured cells. Adult tissues contain large amounts of fully differentiated, post-mitotic cells of a variety of cell types together with their mitotic counterparts, which have undergone different degrees of cellular doublings. On the contrary, replicatively senescent cells represent a single cell type, fibroblasts in our study, almost all being permanently growth-arrested cells. Therefore, aging-associated changes in NF-κB signaling *in vivo* might involve complex mechanisms including many inducers and signaling pathways, which may vary quantitatively and qualitatively between cell types and tissues. Thus, some of these multiple inducers and signals might be absent in replicatively senesced fibroblasts of a single cell type and growing on a monolayer, for example there are no hormonal signals in cell culture. These differences in the cellular environment might be involved in the differences occurring in NF-κB signaling during aging *in vivo* and replicative senescence *in vitro*. As a whole, our results show that age-related changes in NF-κB signaling are, at least partly, differentially regulated during aging *in vivo* and in replicative senescence *in vitro*. This may limit the use of cultured cell models in aging studies involving the NF-κB signaling pathway. However, part of the observed differencies in NF-κB signaling may be due to the differencies in cell types, which exist between animal tissue models and cultured cell models.

6.5 NF-κB and stress response

Free radical reactions and their induced oxidative stress are ubiquitous in all living organisms. Oxidative stress has shown to be one of the most important contributors to the aging process in animal tissues (Harman 1992, Sohal & Brunk 1992). NF-κB is one of the key regulators in cellular defense response to oxidative damage in mammalian cells (Siebenlist et al. 1994). In addition, ROS

are suggested to be common mediators of numerous activation signals in a transient NF-κB activation pathway involving degradation of IκBα (e.g. Piette et al. 1997). However, NF-κB activation pathways, which are independent of ROS production or oxidative stimuli, are also known to exist (e.g. Anderson et al. 1994, Bonizzi et al. 1997).

We used UVB radiation as an inducer of environmental stress to cultured fibroblasts. UVB provides a good model for studying cellular stress responses and their adaptation mechanism to environmental stress (Tyrell 1994). The ability of senescent fibroblasts to respond to this UVB-radiation induced stress was clearly attenuated, suggesting that this activation pathway is affected by age. This attenuated NF-κB activation was further accompanied by a decreased expression of NF-κB target genes indicating that this attenuation may reduce the stress resistance of cells during senescence. The aged cells may, thus, be more vulnerable to environmental stress induced damage. However, the response may be tissue or cell-type specific. A decline in NF-κB signaling with age was also described in murine T cell after CD-3 induction followed by a significant decline in the immune activity of T cells (Trebilcock & Ponnappan 1996). However, the decreasing responsiveness to cytokines with age seems to be independent on the functional activity of the NF-κB signaling pathway in human fibroblasts (Aggarwal et al. 1995). Therefore, UVB-induced protein kinase cascades in NF-κB activation may be distinct from those, which mediate the responses of cytokines to the NF-κB complex.

Our studies with kinase inhibitors (herbimycin A and tyrphostin AG 126) inhibiting the MAPK/JNK pathway (Cox et al. 1996, Takahashi & Berk 1996) showed that both compounds were able to block the UVB radiation induced activation of NF-κB. It seems likely that this signaling pathway is involved in the induction of NF-κB by UVB in fibroblasts. UVB radiation induced also an increase in the AP-1 binding activity, but only in young replicating fibroblasts, suggesting that some of their common upstream regulators may be affected by replicative senescence and be responsible for the observed decrease in their activation. Different MEKK isoforms have been proposed to be involved in signaling responses to different stress stimuli since TNF-α, UV light, and lipopolysaccharides can all activate the JNK pathway (Lee et al. 1998).

6.6 NF-κB and apoptosis

Senescent human fibroblasts are resistant to apoptosis (Wang 1995, 1997), a cellular program which eliminates damaged or unwanted cells. Dr. Eugenia Wang (1997) proposed that the aging associated changes in cells and tissues originates from increased anti-apoptotic resistance of aged cells, which allows damaged and nonfunctioning cells to stay alive in tissues. Accumulation of these kinds of cells in aging individuals may impair proper tissue function and also lead to accumulation of further damage in cellular structures and

functions. With time, this apoptosis resistance, together with the multiple damages, may increase the susceptibility to developing age-dependent diseases.

NF-κB is an interesting transcription factor because it has been reported to control anti-apoptotic responses in some cell types while it is pro-apoptotic in other cells. For example, after forebrain ischemia, the transient activation of NF-κB in most forebrain neurons is proposed to play an anti-apoptotic role and increase the survival by inducing the expression of protective factors, whereas the persistent activation of NF-κB in hippocampal CA1 neurons could have a pro-apoptotic role by inducing the expression of apoptosis inducing proteins and leading to CA1 neuronal death (Clemens et al. 1998). Ewen within a single cell type (in T-cells), NF-κB has been shown to function both a pro-apoptotic regulator (in mature peripheral T cells) and a anti-apoptotic regulatory factor (in thymocytes) (Lin et al. 1999). NF-κB has been shown to be involved in apoptosis inhibition after various signals, for instance, cytokine TNF-α (Beg & Baltimore 1996, van Antwerp et al. 1996), ionizing radiation, the cancer chemotherapeutic drug, daunorubicin (Wang et al. 1996), and Ras induced p53-independent apoptosis (Mayo et al. 1997). Homozygous disruption of the p65 subunit potentiated TNF-mediated apoptosis in liver (Beg et al. 1995), which points to a role for the p65 subunit in this anti-apoptotic mechanism. In addition, the presence of the p65 subunit in the nucleus has been shown to be essential for protection of the photoreceptor cell against apoptosis induced by photo-oxidative stress (Krishnamoorthy et al. 1999). Therefore, the observed age-related increase in the level of nuclear p65 protein together with its increased NF-κB binding activity in liver may be involved in the expression of some apoptosis-related genes with an age-dependent manner protecting aged cells from apoptosis.

NF-κB is required for induction of c-IAP2 gene in suppression of TNF-induced apoptosis (Chu et al. 1997). In addition, c-IAP2 exerts a positive feedback loop in the activation of NF-κB by destabilizing the IκBα inhibitor. Activation of NF-κB also promotes cyclo-oxygenase-2 expression and attenuates apoptotic cell death in activated macrophages (von Knethen et al. 1999). One proposed anti-apoptotic gene (by Baichwal & Baeuerle 1997) is a zinc finger protein A20 (Opipari et al. 1992) expression of which is regulated by NF-κB. Overexpression of p65 protein is also reported to cause G1 arrest and apoptosis in the pro-B cell (Sheehy & Schlissel 1999). In addition, NF-κB has been suggested to promote an apoptotic response in rat striatum through upregulation of c-Myc and p53 (Qin et al. 1999). c-Rel has also been reported to be able to induce apoptosis (Abbadie et al. 1993, Baeuerle & Baltimore 1996). The observed increase in nuclear NF-κB binding activity with age may enhance the expression of some NF-κB target genes, which are involved in the control of apoptosis. The -κB motifs in the promoters of apoptosis-related genes should be studied to determine to what kind of NF-κB complexes they preferably bind and the possible involvement of NF-κB complexes in their expression. The possible influence of NF-κB in the control of apoptosis may also vary from tissue to tissue.

In summary, NF-κB is known to be an important factor controlling apoptosis in cells. The age-related changes in the NF-κB signaling pathway may, thus, be involved in the control of the expression of both anti- and pro-apoptotic genes in aging tissues. Furthermore, the possible NF-κB induced age-related changes in the expression of apoptosis-related genes might be involved in the reported increase in apoptosis resistance, which occurs with age. However, this influence may vary between cell-types and tissues. The role of NF-κB to either promote or attenuate apoptosis most probably depend on the cell type as well as the stimuli, which are the original triggers for the signaling cascade leading to apoptosis.

7 CONCLUSIONS

The main conclusions are:

1. Aging induces up-regulation of constitutive, nuclear NF-κB binding activity in tissues.

2. Aging induces tissue-specific changes in the protein levels of NF-κB components.

3. The observed age-related changes in NF-κB signaling are not due to changes in their inhibitory proteins or the expression levels of NF-κB genes. Instead, these changes are most probably due to the post-translational modification of NF-κB proteins, enhancing either their nuclear localization or the DNA binding activity of NF-κB complex.

4. Aging and replicative senescence attenuate some NF-κB activation pathways and down-regulate the expression of some target genes. However, these changes appear to be highly tissue- and cell-type specific.

5. Age-related changes in NF-κB signaling are differently regulated *in vivo* and *in vitro,* and this will limit the use of cultured cell models in aging studies involving the NF-κB signaling pathway.

6. Age-related changes observed in NF-κB signaling are proposed to be involved in immune senescence and the elevated resistance of apoptosis of aged cells.

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November 1999

YHTEENVETO (RÉSUMÉ IN FINNISH)

Ikääntymisen vaikutus NF-κB:n signalointiin

Kaikki aitotumalliset olennot vanhenevat, ihminen mukaanlukien. Vanhenemiseen liittyviä muutoksia solujen rakenteessa ja toiminnassa tunnetaan monia, mutta niiden syntymekanismit molekyylitasolla ja varsinkin niiden säätely ovat vielä lähes täysin tuntemattomia. Oksidatiivisen stressin ja stressin vastustuskyvyn tiedetään olevan eräitä tärkeimmistä vanhenemiseen liittyvistä tekijöistä. Lisäksi vanhetessa, kuten alkionkehityksen aikana, geenien transkription säätely muuttuu. Tämä viittaa siihen, että transkriptiofaktorit osallistuvat myös vanhenemisen säätelyyn.

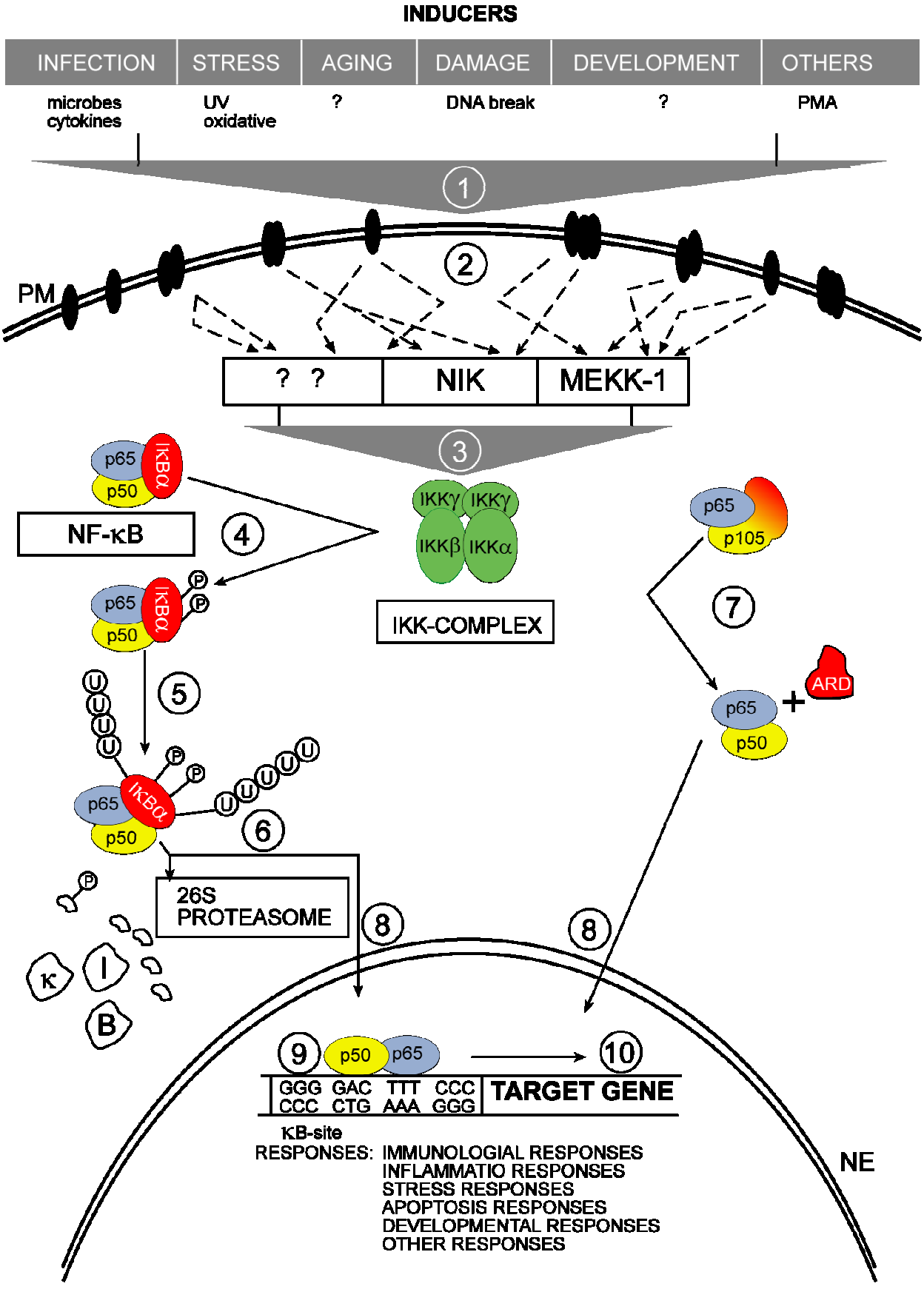
Transkriptiofaktorit ovat proteiineja, jotka säätelevät tarkasti geenien ilmenemistä sitoutumalla geenien säätelyalueisiin. Monet niistä ovat indusoitavia, solun ulkoisten signaalien sensoreita, jotka välittävät tiedon solun ympäristön muutoksesta tumaan ja vaikuttavat siten solussa ilmennettäviin proteiineihin. Solu pystyy näin vastaamaan ympäristönsä muutoksiin. NF-κB signalointireitti on tällainen tärkeä stressivasteen, varsinkin oksidatiivisen stressin, ja erilaisten puolustusreaktioiden välittäjä. Se osallistuu lisäksi mm. kasvun, kehityksen ja apoptoosin säätelyyn ja solujen transformoitumiseen. NF-κB on dimeeri, joka muodostuu sen geeniperheen jäsenten muodostamista erilaisista pareista. Se sijaitsee normaalisti solun sytoplasmassa inhibiittoriinsa sitoutuneena. Aktivaatiosignaali johtaa yleensä inhibiittorin hajoamiseen, jolloin vapautunut NF-κB siirtyy tumaan ja sitoutuu säätelemiensä geenien säätelyalueisiin vaikuttaen näin niiden ilmenemiseen. Poikkeuksia tästä aktivaatioreitistä kuitenkin tunnetaan.

Tässä tutkimuksessa osoitimme, että NF-κB signalointireitissä tapahtuu muutoksia vanhenemisen aikana. Vanheneminen lisää NF-κB:n signaalireitin aktiivisuutta kudoksissa. Nukleaarisen NF-κB:n sitoutumisaktiivisuus lisääntyi voimakkaasti kaikissa vanhojen eläinten kudoksissa. Nousun suuruus vaihteli kudoskohtaisesti. Tähän liittyi myös kudosspesifisiä muutoksia sen alayksiköiden proteiinitasoissa. Sydämessä p52 proteiini lisääntyi vain tumanäytteissä. Maksassa p52 ja p65 proteiinit lisääntyivät sekä tumassa että sytoplasmassa. Aivoissa ei alayksiköissä havaittu kuitenkaan ikämuutoksia. NF-κB signalointireitin säätely näyttäisi siten olevan ikääntymisen aikana kudosspesifistä. Nukleaarisen NF-κB:n sitoutumisaktiivisuus lisääntyminen ikääntymisen aikana saattaa osittain olla seurausta havaituista NF-κB:n proteiinien post-translaationaalisista modifikaatiosta, koska sen inhibiittoreiden proteiinitasoissa ja geeniperheen jäsenten mRNA:n ekspressiotasoissa ei todettu eroja.

Puolustus- ja stressivasteisiin liittyvä NF-κB:n nopea, mutta ohimenevä aktivoitumisreitti oli selvästi hidastunut viljelemällä vanhennetuissa soluissa UVB-induktion jälkeen. Tämän seurauksena sen kohdegeenien ekspressio laski ikääntyneissä, viljellyissä soluissa. NF-κB:n välittämä stressivaste näyttäisi näin alentuneen ja hidastuneen vanhoissa soluissa altistaen niitä soluvaurioille.

Totesimme myös eroja NF-κB reitin ikääntymiseen liityvässä säätelyssä eläinkudosten ja viljelemällä vanhennettujen solumallien välillä, mikä rajoittaa solumallien käyttökelpoisuutta NF-κB signalointireittiin liittyvissä ikääntymistutkimuksissa. Osa näistä muutoksista saattaa johtua solutyyppien eroista, joita on eläinkudosmallin ja soluviljelymallin välillä. Havaitut ikääntymismuutokset NF-κB signaloinnissa aiheuttavat todennäköisesti muutoksia myös joidenkin NF-κB:n kohdegeenien ilmenemisessä ikääntyvissä kudoksissa, missä ne osallistuvat mm. immunologisen puolustusjärjestelmän heikkenemisen säätelyyn ja aiheuttavat muutoksia solujen apoptoosiherkyyden säätelyssä.

Appendix 1: General activation pathway of NF-κB. Diverse groups of inducers can activate NF-κB. (1) Many of them are known to bind to receptors in plasma membrane (PM) triggering a complex cytosolic signal transduction network (2), which is only partially understood today. Many of these signals lead to the activation of the IKK-complex (3). This then phoshorylates inhibitory IκB proteins (4) at specific serine residues triggering their ubiquitination (5) and degradation by 26S proteasome (6). The unbound cytoplasmic NF-κB dimer can then translocate to the nucleus (8). Alternatively, a cytoplasmic NF-κB complex formed of a mature and an inhibitory precursor of NF-κB can be activated by processing the precursor subunit into a mature form (7). Then, the dimer is free to translocate to nucleus (8). In the nucleus, NF-κB dimer can bind to the κB-site in the regulatory regions of target genes (9) enhancing their expression (10). (NE= nuclear envelope, ? = unknown)


INDUCERS
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PM
? ?
NIK
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3
p65
IκBα
p50
NF-κB
4
IKKγ
IKKγ
IKKβ
IKKα
IKK-COMPLEX
P
5
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26S
PROTEASOME
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κ
I
B
p65
p105
7
ARD
9
p50
p65
10
GGG GAC TTT CCC
CCC CTG AAA GGG
TARGET GENE
κB-site
RESPONSES: IMMUNOLOGIAL RESPONSES
INFLAMMATIO RESPONSES
STRESS RESPONSES
APOPTOSIS RESPONSES
DEVELOPMENTAL RESPONSES
OTHER RESPONSES
NE

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ORIGINAL PAPERS

I

Aging-induced up-regulation of nuclear binding activities of oxidative stress responsive NF-κB transcription factor in mouse cardiac muscle

By

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Aging-induced Up-regulation of Nuclear Binding Activities of Oxidative Stress Responsive NF-kB Transcription Factor in Mouse Cardiac Muscle

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M. Helenius, M. Hänninen, S. K. Lehtinen and A. Salminen. Aging-induced Up-regulation of Nuclear Binding Activities of Oxidative Stress Responsive NF-kB Transcription Factor in Mouse Cardiac Muscle. *Journal of Molecular and Cellular Cardiology* (1996) **28**, 487–498. The accumulation of lipofuscin to cardiomyocytes is a classical parameter of aging and is believed to reflect oxidative stress. NF-kB transcription factor complex is one of the cellular sensors which responds to oxidative stress and regulates gene expression. Our purpose was to study whether aging affects the level and distribution of DNA binding activities of NF-kB transcription factors both in cardiac sarcoplasm and nuclear extracts. We used electrophoretic mobility shift assays (EMSA) to characterize the DNA binding activities of NF-kB and two other transcription factors, AP-1 and Sp-1, in the myocardium of 4 months and 24 months old male and female NMRI-mice. The protein levels of p50, p52, and p65 components of NF-kB-complex and an inhibitory IkB-α/MAD-3 were assayed with Western blots. Surprisingly, aging up-regulated by 123% the nuclear NF-kB binding activity in the male and female mice. The sarcoplasmic NF-kB activity, activated by deoxycholate, did not show any change during aging. Aging-induced increase in nuclear NF-kB protein-DNA binding activity was observed both by gel retardation and UV-crosslinking assays. In immunoblotting, the level of p52 component but not those of p50 and p65 components of NF-kB-complex was slightly increased in nuclear fractions. Aging did not affect the sarcoplasmic levels of p50, p52, and p65 proteins. Supershift EMSA assays showed that the nuclear NF-kB complex contained p50, p52, and p65 components. The level of inhibitory IkB-α/MAD-3 protein was unaffected by aging both in nuclear and sarcoplasmic fractions. Aging down-regulated the nuclear Sp-1 binding activities but did not affect AP-1 binding activities. Statistically significant sex-related differences did not appear in the aging responses of transcription factors. These results indicate that NF-kB transcription factor pathway is activated during aging in cardiac muscle and could be the signaling route regulating gene expression. However, the activation mechanism of NF-kB during aging whether oxidative stress responsive or not *in vivo* needs further studies.

Key Words: Aging; Myocardium; Oxidative stress; Transcription factors; NF-kB-pathway; AP-1; Sp-1.

Introduction

The accumulation of lipofuscin to several cell types, especially to cardiomyocytes is a classical aging parameter in animals (Bourne, 1973). Lipofuscin is an end-product of lipid peroxidation reactions induced by reactive oxygen radicals in cells (Sohal and Brunk, 1989). Several lines of evidences show that oxidative stress is one of the most important contributors to the aging process in animal tissues (Harman, 1992; Sohal and Brunk, 1992; Ames *et al.*, 1993). Aging is associated, for instance, with

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reactive oxygen species-induced mutations in genomic (Holmes *et al.*, 1992) and mitochondrial (Richter, 1992) DNA and damages in protein structure (Dean *et al.*, 1992).

Oxidative stress affects the expression of genes and induces the adaptive cellular responses (e.g. Pahl and Baeuerle, 1994). Prokaryotic cells have effective systems to respond to oxidative stress induced by environmental changes (Storz *et al.*, 1990). Eukaryotic cells have also cellular sensors which respond to oxidative stress (Devary *et al.*, 1992; Schreck *et al.*, 1992). One and probably the major signaling system is transmitted by the NF-kB transcription factor pathway (Schreck *et al.*, 1992; Grimm and Baeuerle, 1993; Pahl and Baeuerle, 1994). Cytoplasmic NF-kB transcription factor complex contains normally protein p50/p52 and p65 heteromeric complex which also binds IkB inhibitory protein. This complex is inactive and locates in the cytoplasm because the nuclear localizing peptide signal is covered by inhibitory IkB (Grimm and Baeuerle, 1993). Reactive oxygen responsive protein kinases or kinase cascades phosphorylate the IkB protein and release the transfer of p50-p65-complex to nuclei. The activated complex can bind to the sequence specific promoter and enhancer sites of several genes and regulate the transcription of these genes (Grimm and Baeuerle, 1993).

Several agents inducing oxidative stress, such as hydrogen peroxide (Schreck *et al.*, 1992), oxidized lipoproteins (Andalibi *et al.*, 1993), and ionizing radiation (Brach *et al.*, 1991), activate the cytoplasmic NF-kB complex and increase the nuclear binding activity of NF-kB. The purpose of this study was to find out whether aging affects the nuclear binding activities of NF-kB and whether there are nuclear changes in protein components of NF-kB complex in mouse heart. In addition, we compared the NF-kB binding activities with the binding activities of two other transcription factors, AP-1 and Sp-1.

Materials and Methods

Animals and chemicals

NMRI-mice were obtained from the National Laboratory Animal Center, Kuopio, Finland. Ten male and six female mice, half of them young, 4 months old (weighing 31–48 g), and another half old, 2 years old (weighing 49–59 g) were used in the study. Mice were killed using carbon monoxide. After that hearts were removed, washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at −80°C.

Chemicals and other reagents used were obtained from BDH (Nonidet P40), BioRad (acrylamide, ammonium persulfate, glycine, protein assay reagent, TEMED, Tris base), Boehringer & Mannheim (PMSF), Pharmacia Biotech (poly dI/dC), Promega (AP-1, NF-kB, and Sp-1 oligonucleotides, T4 polynucleotide kinase), Merck (EDTA, glycerol, NaCl), and Sigma (deoxycholic acid, dithiothreitol, Hepes, KCl, $MgCl_2$).

Isolation of proteins

Nuclear proteins were isolated using the modified method of Dignam (Dignam *et al.*, 1983). Frozen hearts were weighed, transferred to Corex tubes, and ice-cold hypotonic buffer (1.5 mM $MgCl_2$, 10mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 mM HEPES, pH 7.9) was added to each sample. The volume was proportioned to the weight of a heart to get 15% homogenates. The tissues were left to thaw on icebath and homogenized (2 × 5 s) using Ultra-Turrax, type 18–10 homogenizer (IKA werk). Homogenates were incubated for 10 min on ice and centrifuged (25 000 × **g**, 15 min, 4°C, RC5C Sorvall Instruments, Du Pont). Cytoplasmic proteins were collected from the supernatants and nuclear proteins from the pellets. One milliliter of supernatant was transferred to a new microfuge tube and centrifuged (15 000 × **g**, 30 min, 4°C, Biofuge A, Heraeus). Supernatants were collected and frozen at −80°C.

Nuclear proteins were isolated from the original pellets. Pellets were washed once with the same volume of hypotonic buffer as was used in the homogenization step and centrifuged (10 000 × **g**, 4°C, 15 min, RC5C Sorvall Instruments, Du Pont). Supernatants were discarded and pellets were suspended in ice-cold low salt buffer (25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 20 mM KCl, 20 mM HEPES, pH 7.9) using the half of the volume of hypotonic buffer. Nuclear proteins were released by adding high salt buffer (25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 1.2 M KCl, 20 mM HEPES, pH 7.9) drop-by-drop using the half of the volume of low salt buffer. Samples were incubated on ice for 30 min. During incubation the tubes were smoothly mixed frequently. Samples were centrifuged (25 000 × **g**, 30 min, 4°C, RC5C Sorvall Instruments, Du Pont) and supernatants were collected in microfuge tubes and stored at −80°C.

The concentration of total proteins in samples were measured using the Protein Assay Reagent of Bio-Rad and their assay protocol.

Labeling of oligonucleotides

Consensus and mutated double-stranded oligonucleotides were purchased from Promega and Santa Cruz companies. The sequences were:
5′AGTTGAGGGGACTTTCCCAGGC-3′ for consensus NF-kB,
5′AGTTGAGGCGACTTTCCCAGGC-3′ for mutated NF-kB,
5′ATTCGATCGGGGCGGGGCGAGC-3′ for consensus Sp-1,
5′ATTCGATCGGTTCGGGGCGAGC-3′ for mutated Sp-1,
5′CGCTTGATGACTCAGCCGGAA-3′ for consensus AP-1, and
5′CGCTTGATGACTTGGCCGGAA-3′ for mutated AP-1.

Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmoles/μl), 1 μl of 10 × kinase buffer, 3 μl of H_2O, 1 μl of T4 polynucleotide kinase, and 3 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) were mixed in a sterile microfuge tube and incubated at 37°C for 20 min. The reaction was stopped by adding 2.5 μl DNA loading buffer (× 10). To separate the labeled probe from the unbound ATP the reaction mixture was loaded to 4% native PAGE gel and run for 90 min with 100 V.

After the gel was run, it was covered with Handi-Wrap and the film (Reflection Nef-496, Du Pont) was exposed for 2–3 min on the top of the gel and developed. The band indicating the labeled probe was cut off and transferred into the Eppendorf tube. The labeled oligonucleotides were eluted out of the gel in 300 μl of distilled H_2O (4°C, overnight). The activity of labeled probe was counted. The probe was diluted using distilled H_2O so that 1 μl contained 10 000 cpm and was stored at −20°C.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were used to characterize the binding activities of NF-kB, Sp-1, and AP-1 transcription factors in nuclear extracts (Ausubel *et al.* 1992). The binding activity of NF-kB was also analysed in cytoplasmic extracts after the activation of inactive cytoplasmic NF-kB with deoxycholate (Baeuerle and Baltimore, 1988). Protein-DNA binding was carried out in a sterile Eppendorf tube. Ten micrograms of nuclear proteins and 25 μg of cytoplasmic proteins were used in each reaction. Because the salt can affect the binding activity (Ausubel *et al.*, 1992), the concentration of salt was counted to the same level in all samples by adding low salt/high salt (2:1) buffer. Salt concentrations were kept low enough not to affect the binding activities. The final volume of each binding reaction was 20 μl. Unspecific binding was blocked using 1 μg of poly dI/dC. In each reaction, 4 μl of five-fold binding buffer (20% glycerol, 5.0 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 5.0 mM DTT, 50 mM Tris/HCl, pH 7.5) was used and 20 000 cpm of labeled probe in 2 μl was added. When analysing the NF-kB binding, 2 μl of 10% Nonidet P40 was added to reaction. To activate the inactive cytoplasmic NF-kB complex, 4 μl of 10% deoxycholic acid was added to the binding assay.

Samples were incubated at room temperature for 15 min. The reaction was stopped by adding 2.5 μl of DNA loading buffer (× 10). The bound and unbound probes were separated in gel electrophoresis (80 V, 90 min, room temperature, 15.17 Vertical Gel Electrophoresis Apparatus, BRL) using 4% native polyacrylamide gel and the running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5). After running the gel was transferred to Watman paper (Watman 3MM) and dried in gel dryer (80°C, 1 h, Model 583 gel dryer, BioRad). The binding results were visualized on an autoradiography film (Reflection Nef-496, Du Pont), which was exposed for 1–2 days at −80°C.

Pilot experiments showed that the NF-kB, Sp-1, and AP-1 binding activities are specific since the mutated binding sites did not bind anything and the use of ten-fold more cold oligonucleotides strongly decreased (over 90%) the binding activities (data not shown).

Supershift experiments to show the components of NF-kB complex were performed by adding 2 μl of the primary antibody to tubes after 10 min binding reaction. Samples were further incubated for 30 min at 4°C. Gel retardation assay was done as described above. Antibodies used were p50 (NL5), p52 (K-27), and c-fos (4) from Santa Cruz and p65 from Boehringer Mannheim.

UV-crosslinking

UV-crosslinking was used to determine the proteins which bind to the kB binding site. The purpose was to analyse the molecular weights of proteins bound to DNA and to compare the binding activities obtained with UV-crosslinking and gel retardation

assays to show the affinity properties of binding complexes. The binding reaction of NF-kB was performed on ice as described above. Crosslinking of proteins to DNA was performed in 96F microwell plates (Nunc) with a UV Stratalinker 1800 (Stratagene) for 15 min on ice. Bound and unbound probes were separated with 10% SDS polyacrylamide gel electrophoresis.

Immunoblotting

Protein components of NF-kB complex were measured in cell extracts with Western blots using the primary antibodies against p50 (NL5), p52 (K-27), and p65 (A) from Santa Cruz. An inhibitory IkB-α protein, was measured using IkB-α/MAD-3 (C-15) primary antibody (Santa Cruz).

Fifteen micrograms of nuclear proteins and 20 μg of sarcoplasmic proteins were mixed with equal amount of 2 × sample buffer (120 mM Tris, pH 6.8, 0.02% bromophenol blue, 20% glyserol, 200 mM DTT, 4% SDS). The prestained SDS-PAGE standard mixture (BioRad) was used as a mw standard in gel electrophoresis. Samples were boiled (3 min, 97°C) and spinned. Proteins were separated in 12% SDS-PAGE gel (running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS). Samples were run with 150V for 4 h.

Proteins were transferred from the gel to a membrane (Immobilon-P, Millipore) using Pharmacia LKB Multiphor II blotting unit and LKB Bromma 2303 Multidrive XL power supply. Current used in blotting was calculated according to the size of the membrane (0.8 mA/cm^2). The blotting time was 1 h and the buffer used was 50 mM Tris, 40 mM glycine, 0.038% SDS, 10% methanol.

For the immunostaining, membranes were blocked (5% fat-free milk in PBS, 0.1% Tween-20 for 1 h at room temperature) and washed (PBS, in room temperature for 2 × 5 min). Membranes were incubated with the primary antibody (1:200 dilution in PBS, 1% BSA, 0.1% Tween-20) for 2 h at room temperature and washed (PBS, 0.1% Tween-20). After washing they were incubated with secondary antibody (goat anti-rabbit HRP, 1:3000 dilution in PBS, 1% BSA, 0.1% Tween-20) for 1 h at room temperature, and washed again (PBS, 0.1% Tween-20, 15 min and 3 × 5 min). Results were visualized on autoradiography film (Reflection Nef-496, Du Pont) using Renaissance Western Blot Chemiluminescence Reagent (Du Pont) according to the protocol of manufacturer.

Statistics

Autoradiographs were analysed using Image Grabber 2.1 (Neotech Ltd.) and Image 1.43 alias programs to measure the mean density of binding bands. Results were analysed with the univariate analysis of variance and non-paired Student's *t*-test.

Results

Aging upregulates the nuclear NF-kB binding activity in heart

Male and female mice were used to measure the age-related changes in nuclear binding activities of transcription factors. Statistically significant sex-related differences, however, did not appear in the responses of NF-kB, Sp-1, and AP-1 transcription factors (Figs 1 and 3, data not shown). Hence the statistical analyses were done with male and female mice together.

A prominent upregulation of nuclear NF-kB binding activity was observed during aging in mouse heart (Fig. 1). The increase in binding activity from 4 to 24 months old mice was 123% ($P<0.05$). Figure 1(a) shows the nuclear NF-kB binding activities of individual male and female mice. The binding of NF-kB (the upper band) is very consistently higher in the hearts of old mice than young adult ones, both in male and female groups.

Instead, the total binding activity of cytoplasmic complexes, activated by deoxycholate, did not show any statistically significant changes during aging [Fig. 1(b)]. However, the storage of NF-kB complexes in cardiac cytoplasm is much higher than the pool in nuclear extracts, so the small decrease observed (17.2%) during aging could reflect the translocation of NF-kB to nuclei although it is not statistically significant due to inter-individual differences.

In order to characterize further the proteins binding to NF-kB site, ultraviolet irradiation was used to crosslink the proteins bound to NF-kB oligonucleotides. Figure 2 shows two major bands about 55–60 kD and one minor band around 100 kD. The major bands most probably represent the complexes of labeled oligonucleotide with p50 or p52 which are the DNA-binding components of NF-kB complex. Interestingly, ultraviolet crosslink experiment also showed a stronger NF-kB binding activity in the nuclear extracts from the hearts of older mice (Fig. 2). Mutated NF-kB oligonucleotides did not bind any proteins (Fig. 2) which verifies the

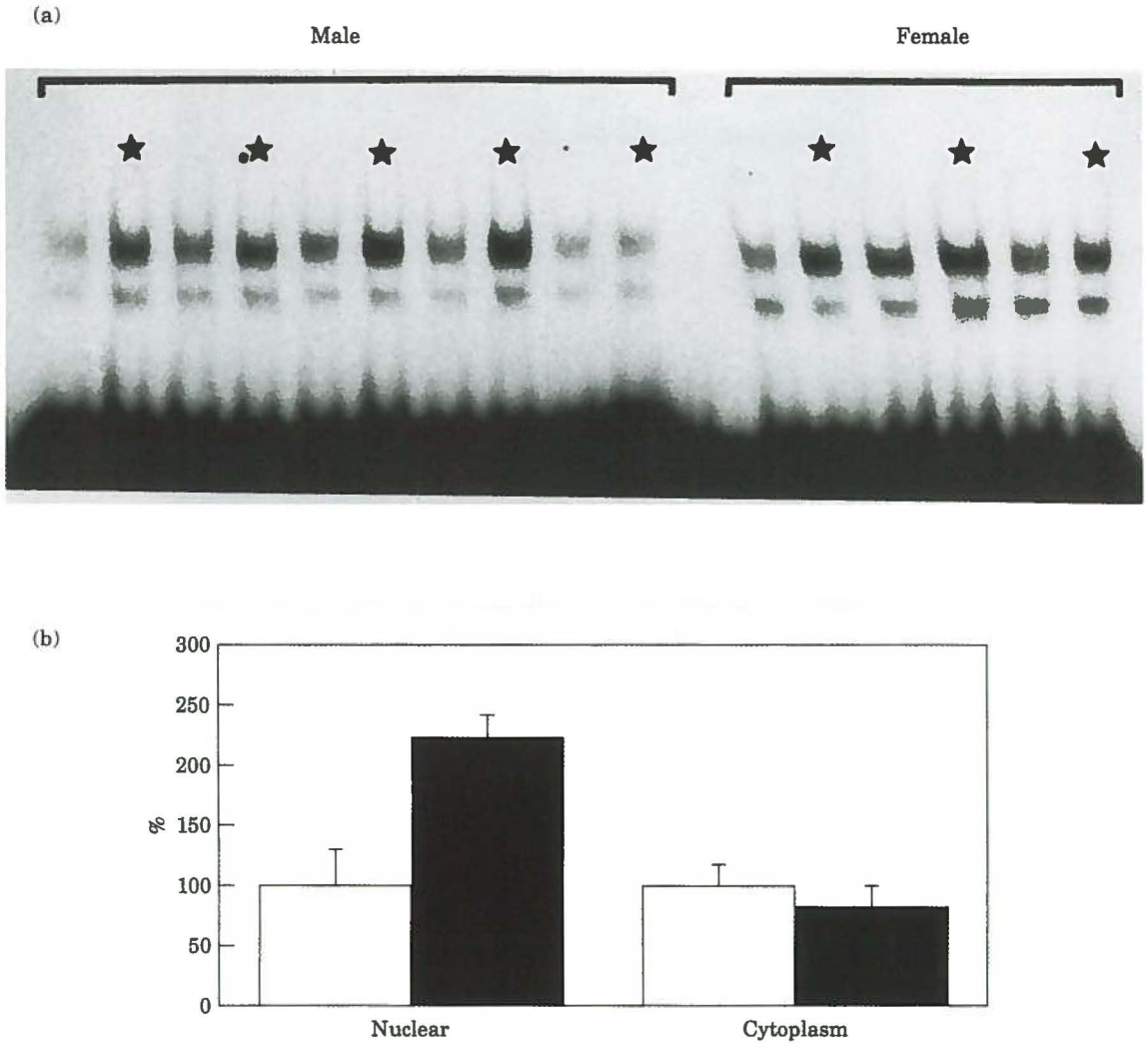

Figure 1 Age-related changes in nuclear and cytoplasmic NF-kB binding activities in mouse cardiac muscle. (a) EMSA binding assays of nuclear NF-kB in individual male and female mice. Old mice are marked with stars. Free unbound probe is on the bottom. (b) Densitometric comparison of nuclear and cytoplasmic NF-kB binding activities in young and old mice. The binding values of male and female mice are combined because there were no sex-related differences. The binding values of young mice are calculated to 100% (+S.E.). Statistical significance: * $P<0.05$ (Nuclear).

results of gel retardation assays (data not shown) and shows the high specificity of binding proteins.

Aging downregulates the nuclear Sp-1 binding activity in heart

The same nuclear protein extracts as used in NF-kB binding assays were also used to assay the binding activities of Sp-1 and AP-1 transcription factors. On the contrary to the NF-kB bindings, the DNA binding activity of Sp-1 transcription factor was considerably lower in the hearts of old mice than in those of young ones (Fig. 3). The age-related difference appeared both in male and female mice [Fig. 3(a)]. The Sp-1 binding activity in the hearts of old mice was only 39.7% ($P<0.05$) of that in young mice [Fig. 3(b)].

The nuclear binding activity of AP-1 transcription factor did not show any statistically significant change during aging in mouse cardiac muscle [Fig. 3(b)]. Inter-individual differences were greater in AP-1 bindings than in those of NF-kB and Sp-1 binding activities.

Protein levels of NF-kB components and inhibitory IkB-α/MAD-3 in heart during aging

Increased nuclear binding activity of NF-kB complex could be due to the increased nuclear translocation

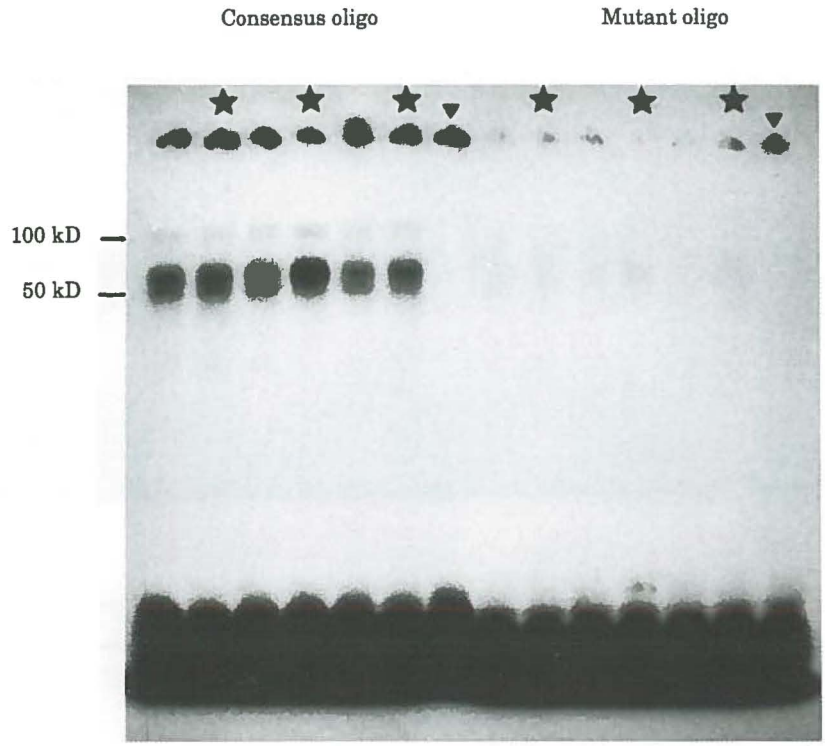

Figure 2 Ultraviolet crosslinking of proteins bound to the NF-kB site. SDS-PAGE gel from the samples of young and old (marked with stars) mice after binding with consensus or mutated oligonucleotides. Lanes with filled triangles show the reactions without nuclear proteins.

and the higher level of protein components in nuclear fraction or to increased binding affinity of protein components due to post-translational modifications. Our experiments using Western blotting of p52 protein component of NF-kB-complex showed that there was no difference between young and old mice in the level of p52 protein expression in cardiac cytoplasm but in nuclear fraction there was a slightly higher level of p52 protein in old mice compared to young ones [Fig. 4(a), (b)]. Statistically, however, the difference was not significant. There was no difference between young and old mice in the nuclear and sarcoplasmic levels of p50 (data not shown) and p65 [Fig. 4(c)] protein components of NF-kB complex.

Supershift assays were used to evaluate the components in nuclear NF-kB complexes. Figure 5 shows that specific antibodies against p50, p52, and p65 but not that against *c-fos* could supershift the NF-kB binding complexes in EMSA assays. The shift of p50 was the strongest and that of p65 was the slightest (Fig. 5). Supershifts were clearly observed in the samples of old mice but overexposure of the film showed very similar supershifts also in the samples of young mice. Antibodies against c-*Rel* and RelB did not induce supershift (data not shown).

Increased NF-kB binding activity during aging could be due to changes in sarcoplasmic IkB proteins which inhibit the translocation of p50/p52 and p65 proteins to nuclei. However, aging did not affect the nuclear or sacroplasmic level of the inhibitory IkB protein α/MAD-3 (data not shown).

Discussion

There are several hypotheses which try to explain aging process at cellular and molecular level (Dice, 1993). The free radical theory of aging, presented by Denham Harman (1956), is nowadays the most recognized from the error theories of cellular aging. Several lines of evidence support the argument that oxidative stress is an important contributor to aging process, especially in post-mitotic cells. For instance, reactive oxygen species-induced DNA-mutations (Holmes *et al.*, 1992; Richter, 1992; Ames *et al.*, 1993) and protein damages (Sohal *et al.*, 1993)

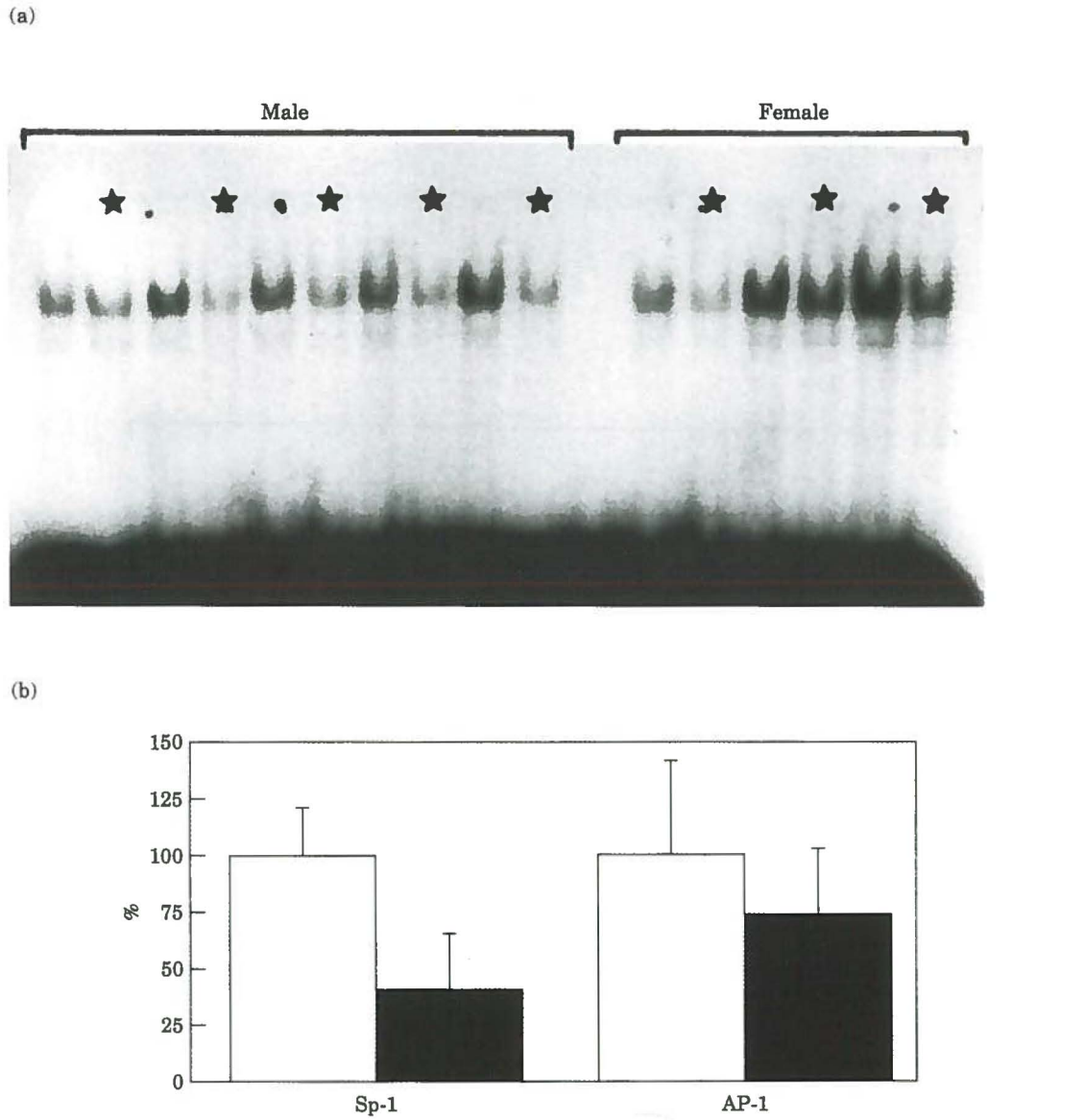

Figure 3 Age-related changes in Sp-1 and AP-1 binding activities in mouse cardiac muscle. (a) EMSA binding assays of Sp-1 in individual male and female mice. Old mice are marked with stars. Free unbound probe is on the bottom. (b) Densitometric comparison of nuclear Sp-1 and AP-1 binding activities in young and old mice. The binding values of male and female mice are combined. The binding values of young mice are calculated to 100% (+S.E.). Statistical significance: * $P<0.05$ (Sp-1).

accumulate during aging into post-mitotic cells. These changes, such as protein oxidative damages, can be associated with life expectancy in houseflies (Sohal *et al.*, 1993). Furthermore, the over-expression of superoxide dismutase and catalase can even extend the life-span in *Drosophila melanogaster* (Orr and Sohal, 1994).

In cardiac muscle, aging increases the rate of mitochondrial production of oxygen radicals which inversely correlates with the life expectancy and maximum life span potential in several mammalian species (Sohal and Brunk, 1992). In human heart, aging increases the accumulation of oxygen damages and deletions in mitochondrial DNA (Hayakawa *et al.*, 1992). In the antioxidant capacity of myocardium, instead, aging-associated changes are surprisingly small (Salminen *et al.*, 1988; Vertechy *et al.*, 1989; Sohal and Brunk, 1992; Vega *et al.*, 1992). Only a slight increase in some enzyme activities, such as catalase (Salminen *et al.*, 1988) and glutathione peroxidase (Vertechy *et al.*, 1989), has been recorded. According to these observations,

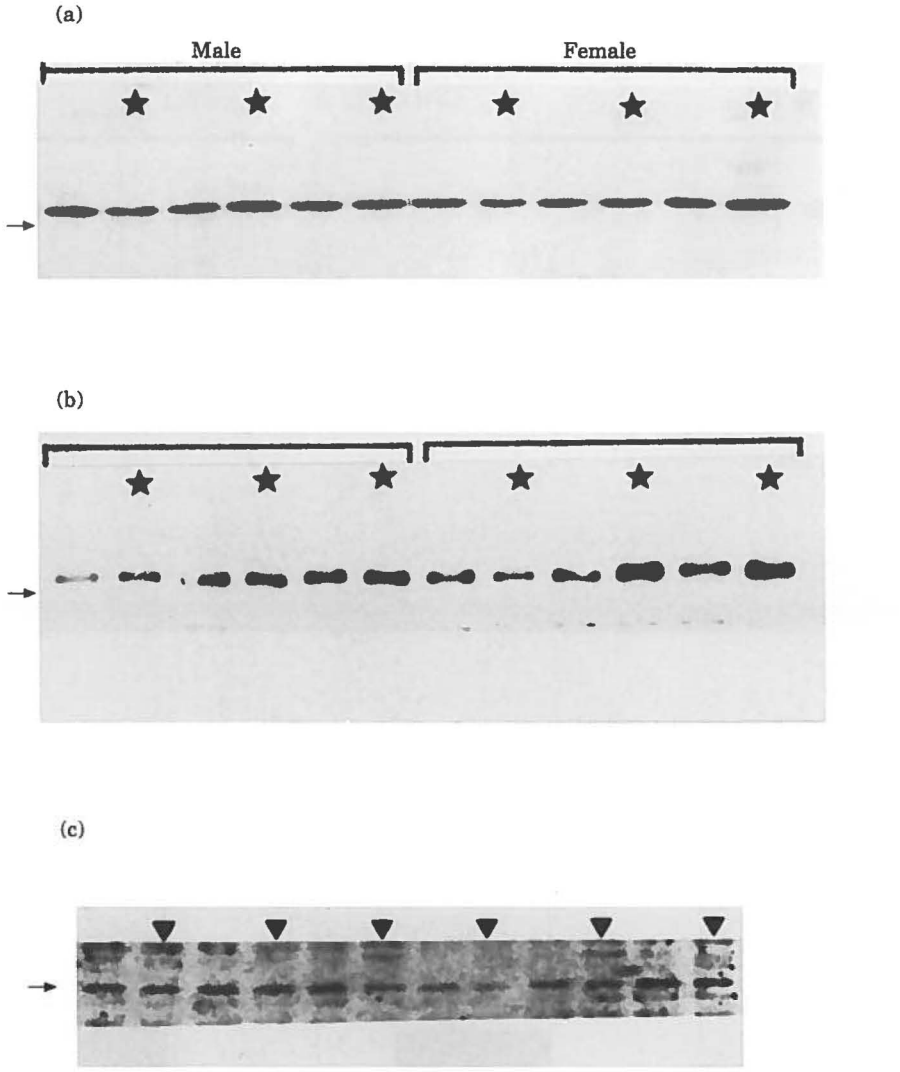

Figure 4 The protein level of p52 component of NF-kB complex in the sarcoplasmic (a) and nuclear (b) fractions in the hearts of young and old mice. Western blot assays of p52 protein are shown in individual male and female mice. Old mice are marked with stars. Arrowheads show the molecular weight of 50 kDa. (c) The protein level of p65 component of NF-kB complex in the nuclear fraction. Old mice are marked with arrowheads. The molecular marker indicates 65 kDa.

there could be a risk to oxygen radicals-induced damages in aging heart.

Mammalian cells have some sensors which respond to changes in cellular redox state. Some of these oxidant sensors are characterized as protein kinases (Bauskin *et al.*, 1991; Devary *et al.*, 1992). Signaling pathways are not known but the nodal point may be the cytoplasmic NF-kB complex (Schreck *et al.*, 1992; Pahl and Baeuerle, 1994). This transcription factor complex which often is a dimer of proteins p65 and p50/p52 is inhibited by IkB proteins in cytoplasm. The activation of NF-kB complex can occur by phosphorylation or proteolytic cleavage (Grimm and Baeuerle, 1993; Henkel *et al.*, 1993). During activation the inhibitory complex will be detached and NF-kB dimer can be translocated to nucleus. In nucleus, the dimers bind to sequence specific promoter and enhancer sites of several genes and regulate transcription of these genes (Grimm and Baeuerle, 1993). Several oxidants increase the nuclear binding activity of NF-kB (Schreck *et al.*, 1992). The increase in antioxidant level, on the contrary, inhibits the cytoplasmic activation and nuclear translocation.

In the present study, we observed a considerable up-regulation of nuclear NF-kB binding activity in mouse heart during aging, especially in gel

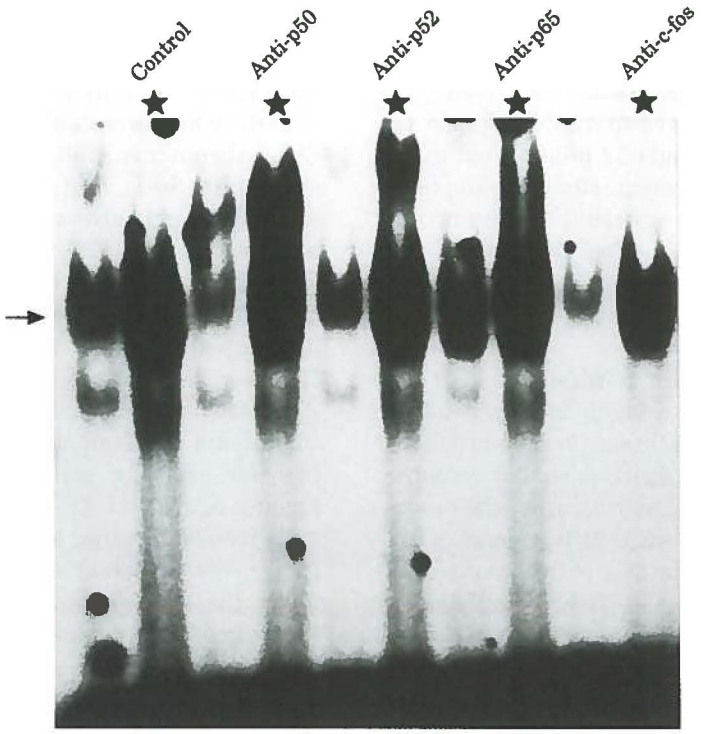

Figure 5 The supershift of NF-kB complex with antibodies against p50, p52, and p65. Young and old (marked with stars) nuclear samples were treated after binding reaction with specific antibodies against p50, p52, p65, and c-*fos* to induce supershift in EMSA assay. Control shows the binding without antibodies. Arrowhead shows the NF-kB binding complex without antibodies bound.

retardation assay. The increase in the nuclear binding activity *in vitro* coincided with the slightly increased nuclear level of p52 protein, one component of NF-kB complex. Sarcoplasmic level of p52 protein was not affected by aging. The increase in the nuclear p52 protein level, however, was markedly less prominent than the increase observed in the binding activities of NF-kB. We could not find any changes in the nuclear and sarcoplasmic levels of p50 and p65 proteins during aging. Supershift assays did not reveal the presence of c-Rel and RelB proteins in NF-kB complexes. Also the immunoblotting of the main inhibitory IkB protein, IkB-α, did not show any age-related difference both in nuclear and sarcoplasmic fractions. Furthermore, deoxycholate treatment did not reveal any additional nuclear NF-kB binding activity which could be due to nuclear inhibitors (data not shown). These observations exclude the possibility that the nuclear localization of NF-kB would be increasingly constitutive in old myocardium due to reduction in synthesis or increase in degradation of IkB proteins (Miyamoto *et al.*, 1994).

As a whole, our results suggest that the increased nuclear NF-kB binding activity could be due either to the sarcoplasmic activation of the NF-kB complex via the phosphorylation and proteolytic cleavage of IkB protein (Grimm and Baeuerle, 1993) or, more probably, to the phosphorylation of the binding proteins themselves because the nuclear protein levels of p50 and p65 were not affected and the level of p52 was only slightly increased. The phosphorylation of p50 (Li *et al.*, 1994) and p65 (Naumann and Scheidereit, 1994) can strongly enhance the binding activity of NF-kB complex. Interestingly, the increase in NF-kB binding activities was stronger in gel retardation assay than in UV-crosslinking assay. Most probably the affinity of NF-kB binding is more critical in gel retardation than UV-crosslinking assays although increased affinity can increase binding activity also in UV-crosslinking assays. Several studies have shown that both of these phosphorylation steps can be induced by oxidative stress activated protein kinases (Anderson *et al.*, 1994; Naumann and Scheidereit, 1994).

The increased binding activity of NF-kB most probably affects the gene expression in the cardiac muscle during aging. The response, however, can be activating or inhibiting due to the components of NF-kB complex bound. Proteins p50 and p52 are DNA binding components but only p65 protein

contains the transactivation domain and hence the dimers between p50 and p52 proteins are inhibitory to transcription (Siebenlist *et al.*, 1994). Our supershift experiment showed that NF-kB complexes were abundant of p50 and p52 proteins but much less abundant of p65 protein. However, the antibody used can affect the supershift results and further experiments with other antibodies are necessary to show the contents of the NF-kB binding complexes before the evaluation of their transcriptional activity.

Our preliminary studies have shown that the binding activity of NF-kB is highly increased during aging also in the mouse brain, liver, and kidney (Helenius *et al.*, unpublished). A similar response to aging in NF-kB binding activity in several tissues prone to aging changes suggests a general mechanism during aging. In the case of liver, our results verify those of Supakar *et al.* (1995) and show a prominent increase in NF-kB binding activity during aging. Interestingly, Supakar *et al.* (1995) observed that NF-kB acts as a negative regulator for the rat androgen receptor gene in liver. Whether increased NF-kB binding activity reflects the inhibitory activity in gene expression, this response could be analogous to the downregulation of the heat shock response, another stress response pathway, during aging (Fawcett *et al.*, 1994; Nitta *et al.*, 1994). However, the downregulation is induced by reducing the heat shock factor binding activity (Fawcett *et al.*, 1994).

Our screening of the literature for the age-associated changes in the expression of genes regulated by NF-kB showed e.g. that the expression of c-*myc* proto-oncogene regulated by NF-kB (Duyao *et al.*, 1990) is progressively upregulated during aging in brain, liver, skin, and heart (Semsei *et al.*, 1989). The activity of heme oxygenase, another NF-kB-regulated target gene (Lavrovsky *et al.*, 1994), increases with aging in rat liver (Bitar and Shapiro, 1987). Interestingly, ferritin H is one of the genes over-expressed in the fibroblasts of Werner's premature aging syndrome (Thweatt *et al.*, 1992). Ferritin H is the target gene for NF-kB transcription factor (Kwak *et al.*, 1995). These few examples show that several target genes for NF-kB are upregulated during aging. Unfortunately, most of the genes known to be regulated by NF-kB have not been studied in heart, especially during aging. However, the nuclear binding activity of NF-kB is surprisingly strong in heart compared e.g. with liver and kidney (Helenius *et al.*, unpublished) which suggests that NF-kB has some important regulatory functions in heart.

Our results showed a prominent decrease during aging in the nuclear binding activity of Sp-1 transcription factor. Ammendola *et al.* (1992) have also observed a strong decrease in the nuclear binding activity of Sp-1 in rat brain and liver during aging. Our experiments (Salminen *et al.*, 1995) have shown that Sp-1, AP-1, and CREB factors are very sensitive to oxidative stress induced by hydrogen peroxide *in vitro*. The inhibition of DNA-binding capacity of these factors is due to the oxidation of SH-groups of cysteine residues in DNA-binding domain (Abate *et al.*, 1990; Myrset *et al.*, 1993). However, there is a difference between Sp-1 and AP-1 factors in respect to the reduction by dithiothreitol and 2-mercaptoethanol after hydrogen peroxide exposure (Salminen *et al.*, 1995). The binding activity of AP-1 could be totally restored with reducing agents but not that of Sp-1 factor. Hence, the oxidation of Sp-1 could permanently inhibit the DNA-binding capacity and downregulate the expression of several house-keeping genes during aging.

As a whole, our observations of aging-associated changes in oxidative stress responsive transcription factors NF-kB and Sp-1 are in agreement with the hypothesis of increased oxidative stress in heart during aging. However, alternative explanations can not be excluded because NF-kB signaling pathway can be activated by several protein kinases, e.g. protein kinase A and C (Shirakawa and Mizel, 1989), and also raf-1 kinase (Li and Sedivy, 1993) which are not oxidative stress responsive protein kinases. Hence, the activation mechanism of NF-kB signaling pathway during aging needs further studies to find out whether the signaling system activating NF-kB is oxidative stress responsive or not *in vivo*.

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II

Age-related changes in the regulation of transcription factor NF-κB in rat brain.

by

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Age-related changes in the regulation of transcription factor NF-κB in rat brain

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Abstract

Aging process involves an increase in stress at cellular level. We studied whether aging affects the regulation of stress responsive transcription factor NF-κB in brain samples of Wistar rats. Hippocampus, cerebellum, and temporal and frontal lobes of cortex were studied. We observed a significant up-regulation in the constitutive, nucleus-located NF-κB binding activity in 30-month-old Wistar rats compared to young and 18-month-old rats. The increase was most prominent in cerebellum and in frontal cortex, but age-related changes did not occur in hippocampus. Inducible, cytoplasmic NF-κB binding activity was not affected by aging in any of the samples studied. Western blot assays did not show any age-related changes in the nuclear level of p50, p52, and p65 protein components of NF-κB complex. Cytoplasmic level of inhibitory IκB-α was also unaffected. The increase in nuclear constitutive NF-κB binding activity during aging may be related to the NF-κB driven cellular response to adapt neurons against apoptotic pressure, as observed recently in several apoptotic conditions. © 1997 Elsevier Science Ireland Ltd.

Keywords: Gene regulation; Electrophoretic mobility shift assay techniques; Apoptosis

Several lines of evidence show that aging is linked to increased level of cellular stress, e.g. oxidative stress [2,11]. In particular, brain tissue is vulnerable, e.g. to oxidant attack that can enhance aging process [10] and contribute to neurodegenerative diseases [1]. Transcription factor NF-κB signaling system is an important stress responsive sensor in mammalian cells [13]. NF-κB transcription factor contains the heterodimeric complex of proteins p50/p52 and p65 [14]. In cytoplasm, NF-κB dimers bind to the proteins of IκB inhibitory family and thus they are inactive. Reactive oxygen species activate redox-state dependent protein kinases which phosphorylate the IκB subunit of the complexes. Active NF-κB complexes are transferred to nuclei where they bind to the sequence specific promoter and enhancer sites of several genes and regulate the transcription of these genes [14].

Proteins of NF-κB transcription factor complexes are widely expressed in neurons and glial cells in brain [8,12]. In neurons, NF-κB complexes are present in cytoplasm in an inducible form and in nuclei in a constitutive form. Inducible cytoplasmic complexes are mostly located at synapses [7]. In rat brain, the highest expression levels of p50 and p65 are present in cerebellum and hippocampus [12]. Several physiological and pathological conditions can affect the expression level of NF-κB subunits in brain [6,12,15]. For instance, the stimulation of ionotropic glutamate receptors activates the NF-κB signaling system [4] and the augmentation of synaptic activity increases the expression of p50 and p65 in neurons [12]. Further, β-amyloid peptide also activates NF-κB signaling pathway, and the expression of p65 protein is up-regulated in hippocampus and cerebral cortex of Alzheimer's disease [15].

We have recently observed that aging strongly increases the nuclear binding activity of NF-κB transcription factor in rat heart and liver [5]. The increase appeared in the constitutive binding activity but not in the inducible cytoplasmic activity. The purpose of this study was to find out whether aging affects the nuclear binding activities of NF-κB in brain and whether there are differences in age-related changes between hippocampus, frontal and temporal cortex, and cerebellum. Furthermore, changes in the nuclear bind-

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ing activities of NF-κB factors were compared to those of protein levels of NF-κB components p50, p52, and p65.

Wistar Hannover rats (National Laboratory Animal Center, Kuopio, Finland) were used in two separate experiments. In the first experiment, female rats were of three ages: young adult (7 months), middle-aged (18 months), and old (30 months). In the second experiment, where different parts of brain were separated, male Wistar Hannover rats of 5 and 30 months were used. Six rats were in each group in both studies. Rats were killed with carbon dioxide. Brain samples were separated and frozen immediately in liquid nitrogen and stored at −75°C.

Tissue samples were homogenized and cytoplasmic and nuclear proteins isolated as we have described earlier in detail [5]. Electrophoretic mobility shift assay (EMSA) was used to assay the DNA binding activity of NF-κB factors. The inducible cytoplasmic binding activity of NF-κB was assayed by adding deoxycholate to the binding reaction [5]. Protein levels of p50, p52, and p65 were analyzed from soluble nuclear proteins and that of IκB-α from cytoplasmic fraction using Western blot method as we have used earlier [5]. The optical densities of DNA-binding complexes were quantified from autoradiographs with an image Grabber 2.1 (Neotech Ltd.) and Image 1.43 program, as done earlier [5].

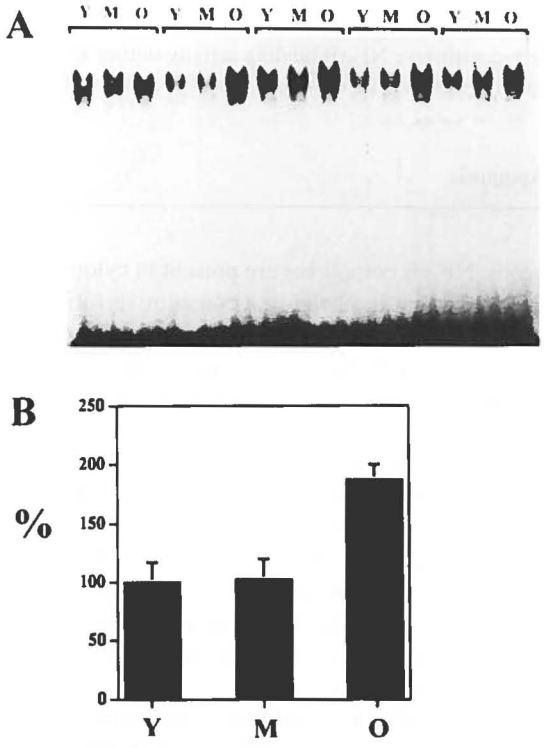

Fig. 1. Age-related increase in the nuclear binding activity of NF-κB in rat brain. (A) EMSA technique shows the differences between young (Y), middle-aged (M) and old (O) rats. Binding activities of five rats in each group have been presented. (B) Densitometric quantification of NF-κB binding activities from (A). The 100% value shows the average value of optical densities of NF-κB complexes for Y rats. The optical density values for M and O rats are compared to the average value of young rats. Variations shown are ± SD. The difference between O and Y/M is statistically significant ($P < 0.01$).

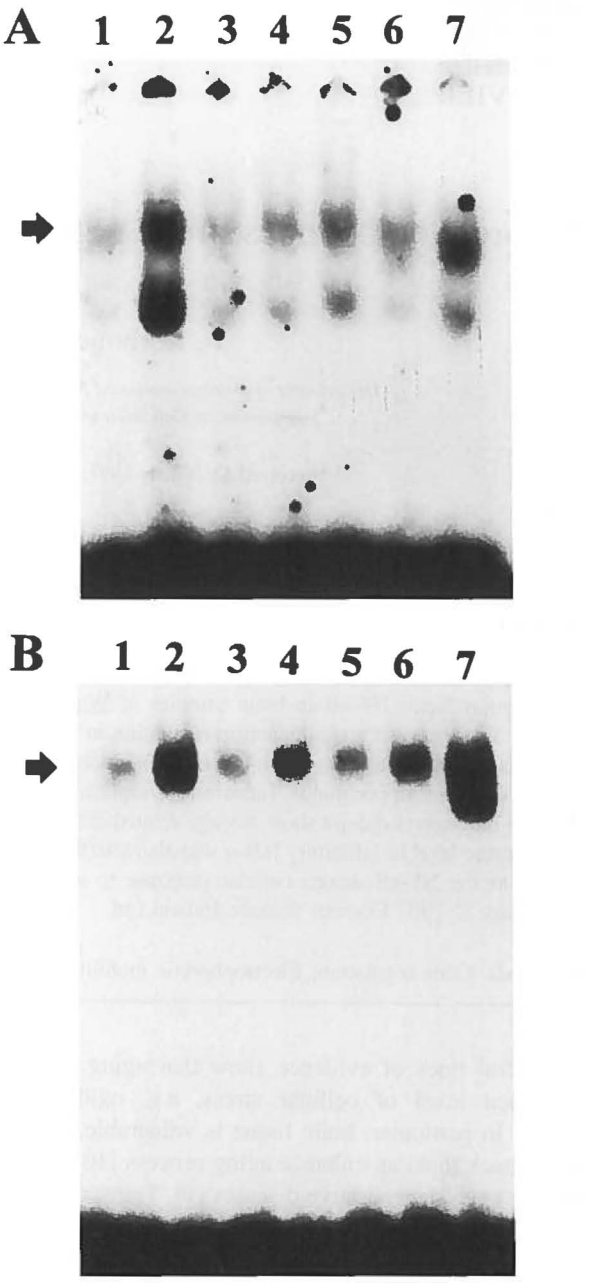

Fig. 2. Regional differences in the constitutive nuclear (A) and inducible cytoplasmic (B) NF-κB binding activities in the brain of young adult (7 months) male Wistar rat. Lanes 1, hippocampus; 2, cerebellum; 3, temporal cortex; 4, frontal cortex; 5, entorhinal cortex and 6, striatum. Lane 7 is liver (medial lobe) for comparison. Arrows show the specific NF-κB binding complex. In binding assays, 10 μg of nuclear protein and 20 μg of supernatant protein was used. Inducible cytoplasmic NF-κB binding activity was assayed by adding 2% deoxycholic acid (final concentration) to binding reaction [13].

Results were calculated using variance analysis and non-paired Student's *t*-test.

Fig. 1A shows that aging induced a consistent increase in the nuclear binding activity of NF-κB in rat brain. The increase did not appear yet in the middle-aged rats but in the oldest, 30-month-old rats (Fig. 1B). These results are very similar to those we observed earlier in heart and liver

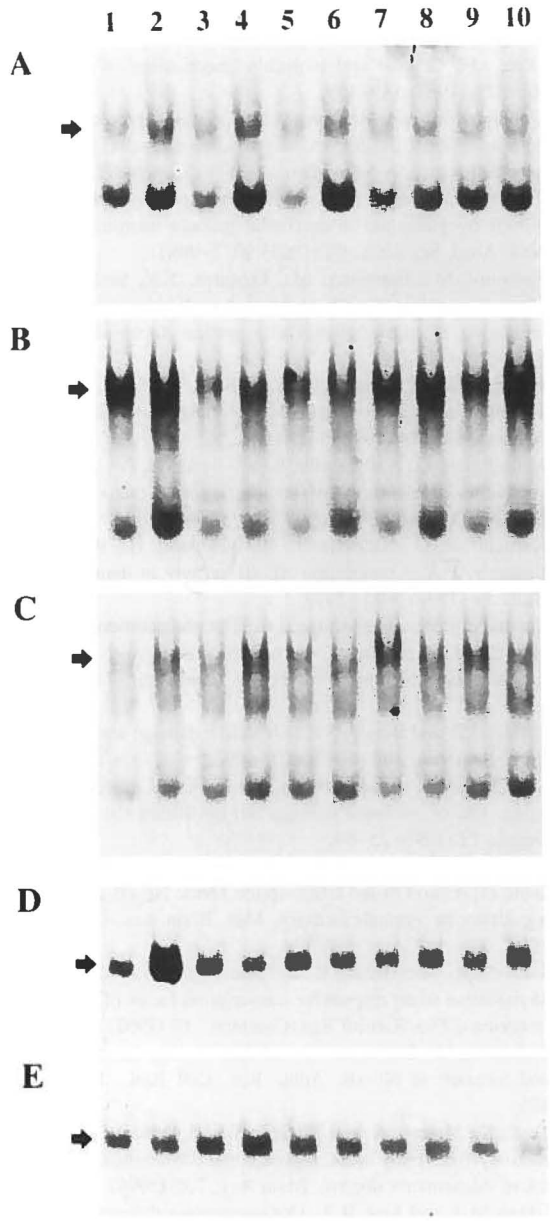

Fig. 3. Regional differences in age-related response in nuclear NF-κB binding activity in rat brain. Figure shows the nuclear binding activities in cerebellum (A), temporal cortex (B) and hippocampus (C), as well as the cytoplasmic binding activities in cerebellum (D) and temporal cortex (E). Lanes 1, 3, 5, 7, and 9 represent 5-month-old male Wistar rats and lanes 2, 4, 6, 8, and 10 represent 30-month-old male Wistar rats. Arrows show the specific NF-κB binding complex.

of the same rats [5]. However, the level of increase in brain was less than in heart and occurred later than in liver.

In the next experiment, we addressed the question whether there are regional differences in the constitutive and inducible binding activities in the brain of young adult rats. Fig. 2 shows that nuclear NF-κB binding activity is the highest in cerebellum (Fig. 2, lane 2), several fold higher than in hippocampus (Fig. 2, lane 1), and in temporal (Fig. 2, lane 3), frontal (Fig. 2, lane 4), and entorhinal cortices (Fig. 2, lane 5), or in striatum (Fig. 2, lane 6). Interestingly, the nuclear NF-κB binding activity in cerebellum is even higher than in liver (Fig. 2, lane 7). Fig. 2B shows that cytoplasmic inducible NF-κB activity is the highest in liver but cerebellum contains a higher inducible NF-κB activity than other brain regions studied. These results are in agreement with those of Meberg et al. [12]. They showed using in situ-hybridization that the expression levels of p50 and p65 were the highest in cerebellum, slightly higher in Purkinje cells than in granule cells [12].

Next, we studied whether there are regional differences in age-related responses in NF-κB binding activities. Fig. 3 shows that statistically significant age-related differences occurred in cerebellum (Fig. 3A) and temporal cortex (Fig. 3B). Instead, age-related differences were not significant in hippocampus (Fig. 3C) or in frontal cortex (data not shown). Age-related increase in cerebellum was 56% which is lower than that observed in whole brain in the first experiment (see Fig. 1). An interesting difference between these two experiments is the sex of the rats used. In the first

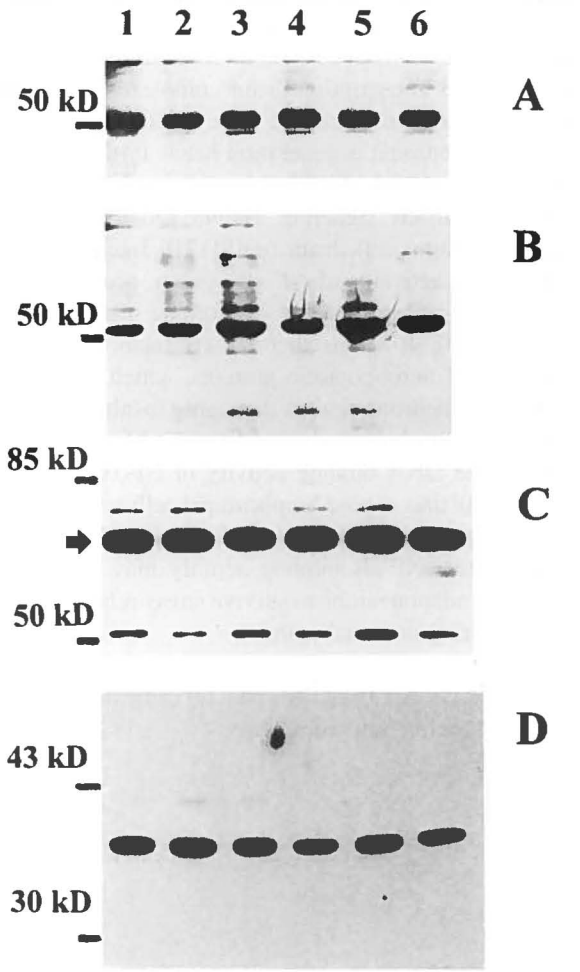

Fig. 4. Aging does not affect the nuclear protein level of p50 (A), p52 (B), and p65 (C), or cytoplasmic level of IκB-α (D) inhibitor protein in rat temporal cortex. Lanes 1, 3, and 5 represent male Wistar rats at the age of 5 months and lanes 2, 4, and 6 those of 30 months old. Molecular weights of 30, 43, 50, and 85 kDa are marked. In (C), the arrow shows the specific immunostaining of p65 proteins.

experiment female Wistar rats were used and in the second one male rats of the same age (see methods). In the cytoplasmic inducible NF-κB activity, we did not observed any statistically significant age-related changes. Results of cerebellum (Fig. 3D) and temporal cortex (Fig. 3E) are shown in Fig. 3.

We have earlier observed that the age-related increase in the nuclear binding activity of NF-κB in rat liver was associated with an increased level of nuclear p52 protein [5]. We studied the nuclear levels of p50, p52, and p65 subunits of NF-κB in rat temporal cortex. Fig. 4 shows that the levels of p50 (Fig. 4A), p52 (Fig. 4B), and p65 (Fig. 4C) did not show significant age-related changes. Furthermore, the cytoplasmic level of IκB-α inhibitory protein was unaffected by aging in temporal cortex (Fig. 4D).

Our present observations confirm that aging causes an increase in constitutive nuclear binding activity of NF-κB also in brain, as it does in heart and liver [5]. However, in brain we could not find any age-related increase in the nuclear level of p52 component of NF-κB as we observed in liver [5]. This suggests that the increase in the binding activity of NF-κB is due to the increase in the binding affinity of NF-κB transcription factor complexes during aging. The functional significance of these results, for instance, in age-related neuronal degeneration needs further studies.

Recently, several studies have shown that the stress responsive NF-κB signaling system provides prevention against apoptotic cell death (e.g. [17]). Interestingly, C2-ceramide, an activator of NF-κB system, protects cultured hippocampal neurons against excitotoxic insult and oxidative stress [3]. It seems that NF-κB factors activate the expression of anti-apoptotic proteins, which increases the resistance of neurons against damaging insults. Glucocorticoids, known to induce neuronal loss in hippocampus [9], attenuate the DNA binding activity of NF-κB in rat brain [16] and may thus expose hippocampal cells to degeneration by reducing anti-apoptotic protection. Age-related increase in constitutive NF-κB binding activity may be a cellular response to adapt neurons to survive stress-related apoptotic pressure during neuronal aging.

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III

Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor -κB

by

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Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-κB

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Both the aging of animals and the senescence of cultured cells involve an altered pattern of gene expression, suggesting changes in transcription factor regulation. We studied age-related changes in transcription factors nuclear factor (NF)-κB, activator protein factor-1 (AP-1) and Sp-1 by using electrophoretic mobility shift binding assays; we also analysed changes in the protein components of NF-κB complex with Western blot assays. Nuclear and cytoplasmic extracts were prepared from heart, liver, kidney and brain of young adult and old NMRI mice and Wistar rats as well as from presenescent, senescent and simian virus 40-immortalized human WI-38 fibroblasts. Aging of both mice and rats induced a strong and consistent increase in the nuclear binding activity of NF-κB factor in all tissues studied, whereas those of AP-1 and Sp-1 decreased, e.g. in liver. Protein levels of p50, p52 and p65 components of the NF-κB complex did not show any age-associated changes in the cytoplasmic fraction but in the nuclear fraction the level of p52 strongly increased in heart and liver during aging. The protein levels of inhibitory IκB-α and Bcl-3 components were not affected by aging in any of the tissues studied. Replicative cellular senescence of human WI-38 fibroblasts induced a strong decrease in nuclear NF-κB, AP-1 and Sp-1 binding activities. Protein levels of p50 and p52 components of NF-κB complex were decreased in the nuclear fraction of senescent WI-38 fibroblasts but in the cytoplasm of senescent fibroblasts the level of p65 protein was increased. Cellular senescence also slightly decreased the protein levels of IκB-α and Bcl-3. Transfection assays with NF-κB-enhancer-driven chloramphenicol acetyltransferase reporter gene showed a significant down-regulation of NF-κB promoter activity in senescent WI-38 fibroblasts. Results suggest that the aging process might be regulated differently in tissues and cultured fibroblasts, perhaps reflecting differences between mitotic and post-mitotic cells. In tissues, aging seems to involve specific changes in the regulation of NF-κB components and perhaps also changes in the DNA-binding affinities of the NF-κB complex.

INTRODUCTION

Aging involves a progressive loss of physiological capacities at both the cellular and tissue levels [1,2]. There are a number of theories of the aging mechanism, which can be roughly divided into error accumulation theories and active programming theories [1]. Research is increasingly focusing on the identification of the genetic basis of age-related repressive gene regulation, which could lead to aging deficiencies in biochemical processes. During development, gene expression is regulated at the level of transcription [3]. The transcription of genes is determined by the combined action of activating and repressive transcription factors that bind to specific sequences in promoter and enhancer regions [4]. The rate of transcription is selectively affected both in senescent cells in culture and in aging tissues *in vivo* [1,5,6].

First evidence now suggests that age-related alterations in the transcription level of various genes could be due to selective changes in transcription factor binding properties and levels of expression during aging [7–10]. Ammendola et al. [7] observed that the binding activity of Sp-1 factor was greatly decreased in the brain and several other tissues in aged rats. The expression of Sp-1 at the protein level, however, was not affected by aging [7]. Dimri and Campisi [8] observed that the binding activities of activator protein factor-1 (AP-1), cAMP-response-element-binding protein (CREB) and CAAT-binding transcription factor complexes to their cognate sequences were decreased in senescent human WI-38 fibroblasts. Senescence-associated changes at the transcription factor binding level turned out to be very selective because the binding activities of octamer-binding protein-B and transcription factor IID were increased and those of nuclear factor (NF)-κB and Sp-1 were unaffected [8]. These observations show examples of the discrepancy in the results between cellular aging *in vivo* and the frequently used *in vitro* aging model, which is based on the Hayflick limit of division capacity of diploid fibroblasts in culture [11].

Several lines of evidence show that aging is associated with increased levels of oxidative stress [12–14]. We were interested to study whether aging also affects the regulation of the oxidative-stress-responsive transcription factor NF-κB [15]. Here we report that aging strongly increased the nuclear binding activity of NF-κB factor but not those of Sp-1 and AP-1, which mostly decreased or were unaffected during aging in rat and mouse tissues. By contrast, in cultured senescent fibroblasts the nuclear binding activities of all transcription factors studied consistently decreased. Further studies on the regulation of the NF-κB complex showed that, in animal tissues, aging selectively increased the nuclear level of the p52 component of the NF-κB complex. These results show that aging specifically modifies transcription factor binding activities and that aging is regulated differently in tissues and cultured fibroblasts. However, the aging response could be repressive to the function of the NF-κB signalling system in both tissues and cultured fibroblasts.

Abbreviations used: AP-1, activator protein factor-1; CAT, chloramphenicol acetyltransferase; CREB, cAMP-response-element-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift binding assay; NF, nuclear factor; SV, simian virus.

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MATERIALS AND METHODS

Animals and materials

Wistar Hannover rats and NMRI mice were obtained from the National Laboratory Animal Center (Kuopio, Finland). Male ($n = 10$) and female ($n = 6$) mice, half of them young adults (4 months old) and the other half old (2 years old) were used in the study. Female rats were of three age categories, with six animals in each group: young adult (7 months), middle-aged (18 months) and old (30 months). Animals were killed with carbon dioxide. Tissues were removed, washed in ice-cold 0.9 % NaCl, frozen in liquid nitrogen and stored at −80 °C.

Double-stranded oligonucleotide-binding sites to NF-κB, AP-1 and Sp-1, as well as the T4 polynucleotide kinase, were from Promega. Poly(dI-dC)·poly(dI-dC) was obtained from Pharmacia Biotech. PAGE chemicals and protein assay reagent were from BioRad. Primary antibodies against p50 (NLS), p52 (K-27, D-32), IkB-α/MAD-3 (C-15) and Bcl-3 (C-14) were from Santa Cruz and that against p65 (14631A) was from Pharmingen.

Cell culture

Human lung WI-38 fibroblasts were obtained from the American Type Culture Collection. The passage number was 15 when we obtained the fibroblasts. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and the antibiotics penicillin and streptomycin. Fibroblasts were replicatively senesced by culturing up to passage 35 before being used in the assays.

Isolation of proteins

Nuclear proteins were isolated by the modified method of Dignam [16], described earlier in detail [17,18]. Tissues prepared for experiments were the cortices of brain, medial lobe of liver, right kidney and cardiac ventricles. Frozen tissues were weighed, transferred to Corex tubes and homogenized with an Ultra-Turrax homogenizer in ice-cold hypotonic buffer [1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 1.0 mM dithiothreitol (DTT), 10 mM Hepes, pH 7.9]. Homogenates were incubated for 10 min on ice and centrifuged (25000 *g*, 15 min, 4 °C; RC5C Sorvall Instruments, Du Pont). Cytoplasmic proteins were collected from the supernatants and nuclear proteins from the pellets. Pellets were washed once and centrifuged at 10000 *g* for 15 min at 4 °C after which pellets were suspended in ice-cold low-salt buffer [25 % (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 20 mM KCl, 20 mM Hepes, pH 7.9]. Nuclear proteins were released by adding a high-salt buffer (25 % glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 1.0 mM DTT, 1.2 M KCl, 20 mM Hepes, pH 7.9) drop by drop to a final concentration of 0.4 M KCl [16,17]. Samples were incubated on ice for 30 min, with smooth shaking. Soluble nuclear proteins were recovered by centrifugation (25000 *g*, 30 min, 4 °C) and proteins were stored at −80 °C. The concentration of total protein in samples was measured with the Protein Assay Reagent kit of Bio-Rad.

Electrophoretic mobility shift assay (EMSA)

The EMSA method was used to characterize the binding activities of NF-κB, Sp-1 and AP-1 transcription factors in nuclear extracts [19]. The binding activity of NF-κB was also analysed in cytoplasmic extracts after the activation of inactive cytoplasmic NF-κB with deoxycholate [20].

Consensus and mutated double-stranded oligonucleotides of NF-κB, AP-1 and Sp-1 were labelled with T4 polynucleotide kinase by using the protocol of Promega. Labelled oligonucleotides were purified with native 4 % PAGE and dissolved from the gel to distilled water by overnight incubation at 4 °C.

Protein–DNA binding assays were performed with 10 μg of nuclear protein and 25 μg of cytoplasmic protein in each reaction. Because salt can affect the binding activity [19], the concentration of salt was adjusted to the same level in all samples by adding low-salt and high-salt buffer. Unspecific binding was blocked by using 1 μg of poly(dI-dC)·poly(dI-dC). The binding medium contained 4 % glycerol, 1.0 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 1.0 mM DTT and 10 mM Tris/HCl, pH 7.5. In each reaction 20000 c.p.m. of radiolabelled probe was included. In NF-κB binding assays, 1.0 % (v/v) Nonidet P40 was included. To activate the inactive cytoplasmic NF-κB complex, 2.0 % (w/v) deoxycholic acid was included in the binding assay. Samples were incubated at room temperature for 15 min. Bound and unbound probes were separated in a native 4 % polyacrylamide gel with a running buffer of 50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5. Binding results were revealed on an autoradiography film (Reflection Nef-496, Du Pont) exposing for 1–2 days at −80 °C.

Western blot assay

NF-κB protein components were assayed in cell extracts with Western blot assays by using primary antibodies from Santa Cruz against p50 (NLS), p52 (K-27 and D-32), IκB-α/MAD-3 (C-15) and Bcl-3 (C-14) and against p65 (14631A) from Pharmingen. A 15 μg portion of each protein sample was separated in SDS/PAGE (12 % gel) with a running buffer of 25 mM Tris, 192 mM glycine and 0.1 % SDS. Prestained SDS/PAGE standard mixture (Bio-Rad) was used as a molecular mass standard.

Proteins were transferred from the gel to membrane (Immobilon-P; Millipore) with a Pharmacia LKB Multiphor II blotting unit and a buffer of 50 mM Tris, 40 mM glycine, 0.038 % SDS and 10 % (v/v) methanol. Membranes were blocked [5 % (w/v) fat-free milk in PBS containing 0.1 % Tween-20 for 1 h at room temperature] and incubated with the primary antibody [1:200 dilution in PBS containing 1 % (v/v) BSA and 0.1 % Tween-20] for 2 h at room temperature. The secondary antibody used was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000 dilution in PBS containing 1 % BSA and 0.1 % Tween-20) for 1 h at room temperature. Results were revealed on autoradiography film by using Renaissance Western Blot Chemiluminescence Reagent (Du Pont) in accordance with the manufacturer's protocol.

Transfections

Young (passage 20) and senescent (passage 33) human WI-38 fibroblasts were transiently transfected with plasmid NFκB-TK10-CAT, which contains a mouse TNF-α κB enhancer and chloramphenicol acetyltransferase (CAT) reporter gene [21]. Fibroblasts were co-transfected with pCH110 (Amersham) containing the *lacZ* reporter gene to exclude differences in transfection efficiences and translation capacities between young and senescent WI-38 fibroblasts. Transfections were made with the calcium phosphate precipitation method and proteins were harvested after 48 h. The expression of CAT was analysed with a CAT-ELISA kit (Boehringer) and that of β-galactosidase by using chlorophenol red-fA-D-galactopyranoside substrate (Boehringer). Assays were made in accordance with the manufacturer's protocol.

Statistics

The autoradiographs were analysed with an Image Grabber 2.1 (Neotech Ltd.) and Image 1.43 programs to measure the mean densities of binding bands. Results were analysed with a univariate analysis of variance and the non-paired Student's t test.

RESULTS

Up-regulation of nuclear NF-κB binding activity in tissues during aging

Transcription factors AP-1, Sp-1 and NF-κB showed a variable binding level in different tissues and specific changes during aging. Most interestingly, a strong and consistent up-regulation of nuclear NF-κB binding activity was observed during aging in all tissues studied, in both rats and mice (Figure 1, and results not shown). The up-regulation of this oxidative-stress-responsive transcription factor was most marked in liver and heart. The increase in the nuclear binding activity of NF-κB seemed to coincide with the aging process, because an increased level of nuclear NF-κB binding was already observable in middle-aged rats (Figure 1). The increase in binding activity from 4- to 24-month-old mice varied from 45 % in brain to 120 % in liver. In rat, the highest age-related increase occurred in heart, where the change was 3-fold (Figure 1B). All these changes were statistically significant ($P < 0.01$). However, the total binding activity of the cytoplasmic NF-κB complex, activated by deoxycholate, did not show any statistically significant changes during aging in any of the tissues studied. Figure 1D shows the binding activities in mouse liver.

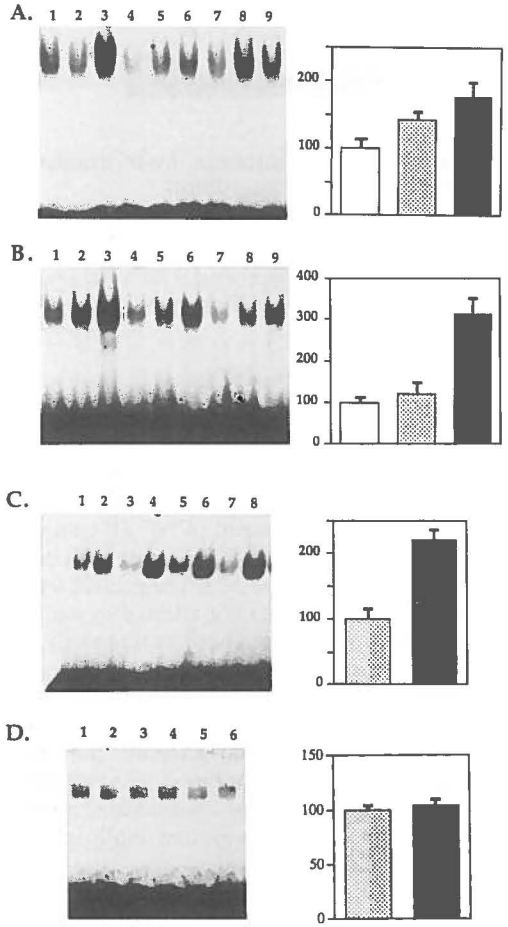

Figure 1 Aging-induced up-regulation of NF-κB binding activity in rat and mouse tissues

The EMSA method was used to compare nuclear NF-κB-binding activities in different tissues of rats and mice. Rat liver (**A**) and heart (**B**) samples of young adult (lanes 1, 4 and 7), middle-aged (lanes 2, 5 and 8) and old (lanes 3, 6 and 9) rats are shown on the left and the corresponding densitometric values from all animals in each group ($n = 6$) are presented on the right. (**C**) Mouse liver nuclear samples of young adult (lanes 1, 3, 5 and 7) and old (lanes 2, 4, 6 and 8) mice. (**D**) Mouse liver cytoplasmic samples of young adult (lanes 1, 3 and 5) and old (lanes 2, 4 and 6) mice. NF-κB-binding activity was activated by deoxycholate (see the Materials and methods section). On the right are densitometric results of eight young (grey column) and eight old (black column) mice. The values for young adult rats and mice are calculated to 100%; variations shown are S.D. All nuclear NF-κB differences between young adult and old animals are statistically significant ($P < 0.01$).

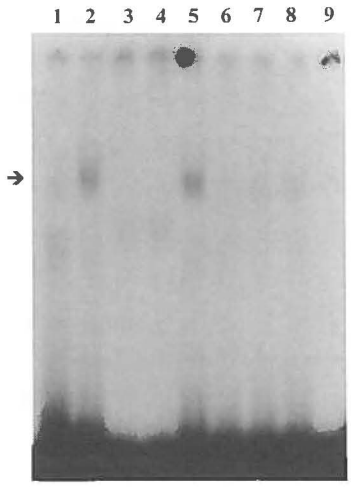

Figure 2 Effects of mutation of the NF-κB binding site and the addition of competing unlabelled consensus oligonucleotides on NF-κB binding in rat liver

The EMSA method was used to compare the nuclear NF-κB-binding activity of liver to consensus oligonucleotides in young (lane 1) and old (lane 2) rats, as well as to mutated oligonucleotides in young (lane 3) and old (lane 4) rats. The addition of competing unlabelled consensus oligonucleotides to the NF-κB-binding reaction of old rat liver: no addition (lane 5); 100-fold (lane 6), 50-fold (lane 7) and 10-fold (lane 8) excess. Lane 9, probe without protein sample. The arrow shows the NF-κB binding.

All age-related changes in the nuclear NF-κB binding activity were quantitative because any new binding complexes could not be observed with the PAGE retardation method used (Figure 1). Furthermore we studied the specificity of NF-κB binding by using the mutated binding site (Santa Cruz) and competing unlabelled oligonucleotides. These experiments showed that NF-κB-binding activity is specific because the mutated NF-κB-binding site does not bind the complex, and the addition of 10-fold unlabelled oligonucleotides to the binding reaction strongly decreased the NF-κB binding activity (Figure 2).

To compare the highly increased nuclear NF-κB binding activity with other transcription factors we also analysed the binding activities of AP-1 and Sp-1 from the same nuclear extracts. In agreement with the observations of Ammendola et al. [7], the binding activity of Sp-1 was strongly down-regulated during aging in the brain and heart of mice and in the liver and heart of rats. In rat brain, however, there was no age-related difference in the binding activity of Sp-1 (results not shown). The binding activity of AP-1 showed a high inter-individual variance and the only statistically significant age-related alteration observed was the 60 % reduction in mouse liver (results not shown).

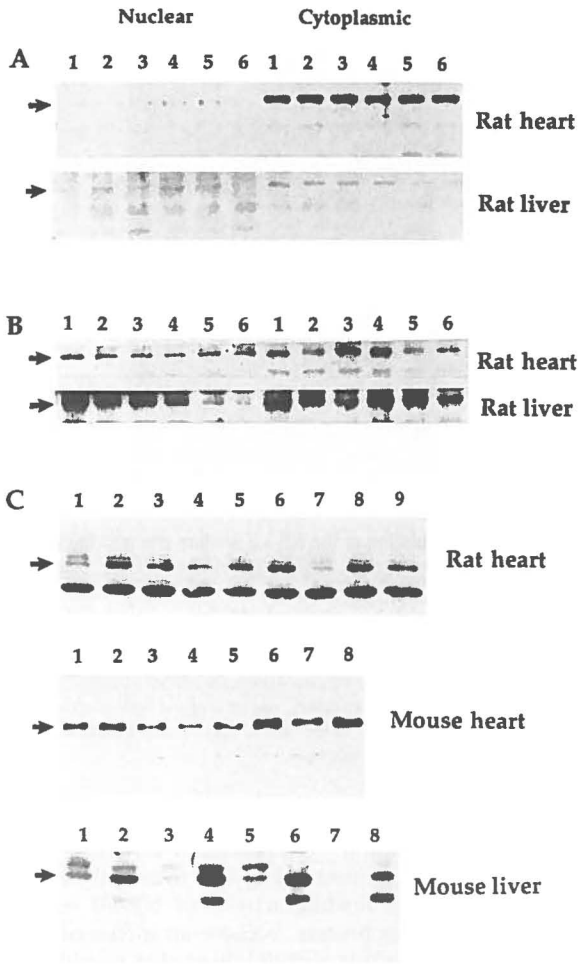

Figure 3 Protein levels of NF-κB components in tissues of mice and rats of different ages

Western blot assays of p65 (**A**), p50 (**B**) and p52 (**C**) protein components of the NF-κB complex in nuclear and cytoplasmic fractions of rat and mouse heart and liver. (**A**) Nuclear and cytoplasmic samples of rat liver and heart of young adult (lanes 1 and 4), middle-aged (lanes 2 and 5) and old (lanes 3 and 6) animals. (**B**) Nuclear and cytoplasmic fractions of rat heart and liver of young adult (lanes 1 and 4), middle-aged (lanes 2 and 5) and old (lanes 3 and 6) animals. (**C**) Nuclear fractions of the heart of young adult (lanes 1, 4 and 7), middle-aged (lanes 2, 5 and 8) and old (lanes 3, 6 and 9) rats. Below are the nuclear fractions of mouse heart and liver of young adult (lanes 1, 3, 5 and 7) and old (lanes 2, 4, 6 and 8) animals. Arrows indicate the positions of the specific proteins.

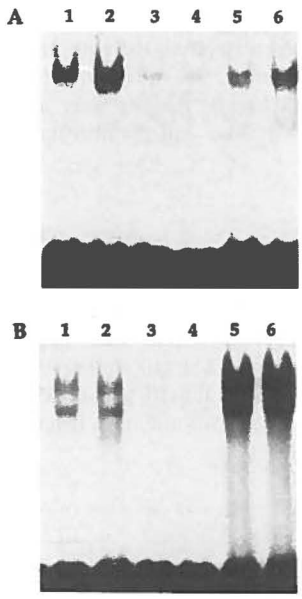

Figure 4 Effects of replicative senescence, SV-40 transformation and quiescence on NF-κB and Sp-1 binding activities

The EMSA method was used to compare nuclear NF-κB (**A**) and Sp-1 (**B**) binding activities in proliferating (lane 1), senescent (lane 3) and SV-40-transformed (lane 5) human WI-38 fibroblasts and corresponding quiescent fibroblasts (lanes 2, 4 and 6). The quiescence of young, senescent and SV-40-transformed fibroblasts was induced by culturing cells in 0.2% fetal calf serum for 72 h [8].

Changes in protein components of NF-κB complex during aging

The increase in the binding activity of nuclear NF-κB observed during aging could be due to changes in constitutive protein levels of NF-κB components, changes in the binding affinity of the components or changes in the distribution of NF-κB components between cytoplasm and nuclei. We analysed the protein levels of p50, p52 and p65 as well as inhibitory IκB-α and Bcl-3 by Western blot immunostaining in nuclear and cytoplasmic samples of liver and heart tissues from mice and rats. Figure 3(A) shows that the p65 component of NF-κB complex is present mostly in cytoplasm, and age-related differences were not observed in either the total protein levels or the distribution. The protein level of p50 was more abundant and more evenly distributed between nuclei and cytoplasm than the p65 component (Figure 3B). Age-related alterations were not observed in any tissue.

The amount of the p52 component of NF-κB complex in the nuclear fraction increased strongly during aging both in the heart and liver of mice and rats (Figure 3C). The protein level of p52 in the cytoplasm was low and was not affected by aging (results not shown). In the liver of old animals, there frequently appeared a low-molecular-mass immunopositive band, which could be a proteolytic fragment of p52 protein (Figure 3C).

The protein levels of inhibitory IκB-α and Bcl-3 were not affected by aging in any tissue studied (results not shown). In heart, IκB-α was nearly evenly distributed between the nuclear and cytoplasmic fractions but in liver it was located mainly in the cytoplasm. The inhibitory Bcl-3 was located totally in the nuclear fraction both in liver and heart.

Replicative senescence of human WI-38 fibroblasts in culture

Replicative senescence of WI-38 fibroblasts strongly decreased the proliferative capacity of cells and induced morphological changes, such as flatness of fibroblasts. In the cellular senescence experiments we compared the results of aging with those observed in simian virus (SV)-40-immortalized WI-38 fibroblasts. In contrast with the aging-induced up-regulation of NF-κB binding activity in tissue, in senescent fibroblasts the nuclear binding activity of NF-κB was strongly decreased (Figure 4). The quiescence of the growth potential of WI-38 fibroblasts by serum deficiency surprisingly showed an up-regulation of NF-κB-binding activity in both young proliferative and SV-40 immortalized fibroblasts, but not in quiescent senescent WI-38 fibroblasts

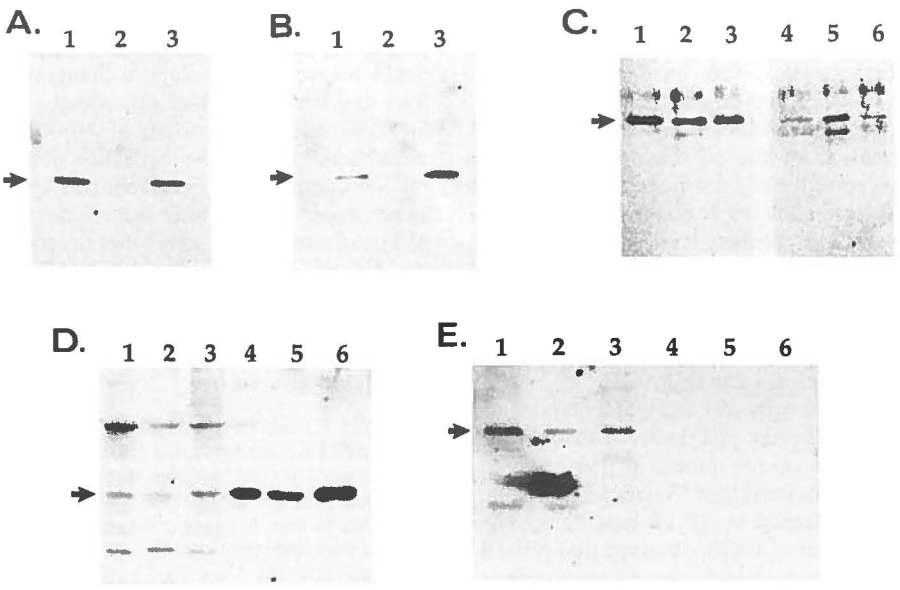

Figure 5 Protein levels of NF-κB components in proliferating, senescent and SV-40-immortalized fibroblasts

Western blot assays of protein levels of p50 (**A**), p52 (**B**), p65 (**C**), IκB-α (**D**) and Bcl-3 (**E**) components in nuclear and cytoplasmic fractions of human WI-38 fibroblasts. Lanes 1, 2 and 3, nuclear fractions of young, senescent and SV-40-immortalized fibroblasts respectively; lanes 4, 5 and 6, cytoplasmic fractions of young, senescent and SV-40-immortalized fibroblasts respectively. Arrows show the positions of the specific protein.

(Figure 4). This phenomenon appeared only with NF-κB-binding activity but not with Sp-1-binding activity (Figure 4). The nuclear binding activity of Sp-1 was strongly up-regulated by SV-40 immortalization and down-regulated by aging (Figure 4).

Western blot assays of the NF-κB components showed that nuclear levels of p50 (Figure 5A) and p52 (Figure 5B) proteins were strongly decreased in senescent WI-38 fibroblasts, but that of p65 (Figure 5C) decreased only slightly. Surprisingly, the level of cytoplasmic p65 protein was up-regulated in senescent WI-38 fibroblasts (Figure 5C). Cytoplasmic levels of p50 and p52 proteins were low and not affected by replicative senescence (results not shown).

The inhibitory IκB-α was located mostly in the cytoplasm (Figure 5D) but Bcl-3 was present only in nuclei (Figure 5E). Senescence of WI-38 fibroblasts decreased the levels of inhibitory IκB-α and Bcl-3 proteins to some extent.

Transfection assays with the NF-κB enhancer-driven CAT reporter gene plasmid were performed to show whether the decreased binding activity of NF-κB and the decreased protein levels of p50 and p52 affect the NF-κB-driven promoter activity. Young (passage 20) and senescent (passage 33) WI-38 fibroblasts were co-transfected with NFκB-TK10-CAT and pCH110 plasmids (see the Materials and methods section), in four separate experiments with each category of fibroblast. In young cells the promoter activity was 165 ± 38 pg of CAT per unit of β-galactosidase; in senescent cells the activity was 106 ± 14 (means $\pm$ S.E.M.; $P < 0.01$). These results show a significant down-regulation of the NF-κB-mediated promoter activity in senescent WI-38 fibroblasts.

DISCUSSION

Aging of tissues *in vivo* as well as the replicative senescence of cultured cells affect the expression patterns of a number of genes [1,2,6]. Several lines of evidence suggest that aging could be due to programmed changes in gene expression [1,6]. Programming could occur via the modulation of the profile and the function of transcription factors. However, there have been few studies on the expression level and the binding activities of transcription factors during aging and in replicative senescence [7–10,21].

Ammendola et al. [7] observed a strong and consistent decrease in the binding activity of Sp-1 in rat tissues such as brain and liver. In agreement with the observations of Ammendola et al. [7] we observed a strong down-regulation of Sp-1 in mouse brain and heart and in rat liver and heart. We could not find in rat brain any age-related differences in Sp-1 binding activity. Ammendola et al. [7] reported that aging does not affect the protein level of Sp-1 although the binding activity is markedly decreased. The binding activity of Sp-1 could be decreased by several regulatory mechanisms. We have observed that Sp-1 as well as AP-1 and CREB binding factors are sensitive to oxidative stress induced by the treatment of nuclear brain extracts with hydrogen peroxide *in vitro* [17]. The inhibition of DNA-binding activity of these factors is due to the oxidation of the thiol groups of cysteine residues in the DNA-binding domain [23,24]. The reduction of oxidized proteins with DTT and mercaptoethanol could totally restore the binding activities of AP-1 and CREB factors, but not Sp-1 binding [17]. Hence the oxidation of Sp-1 factor could permanently inhibit the DNA-binding activity of this factor and down-regulate the expression of several housekeeping genes that contain GC-boxes during aging.

The nuclear DNA-binding activity of the NF-κB factor strongly increased during aging in all tissues of rats and mice studied. This is in contrast with the down-regulation of the binding activity of Sp-1 and also that of AP-1 in some tissues. The increase in nuclear NF-κB-binding activity could be observed in, for example, liver tissue even in middle-aged rats, which shows that the process coincides with aging. There are several possible reasons why the nuclear binding activity of NF-κB could increase during aging. The most frequent cause of nuclear up-regulation is the activation of an inactive NF-κB complex in the cytoplasm and the translocation of NF-κB dimers to the

nuclei [25]. This seems unlikely during aging, however, because the protein levels of cytoplasmic p50, p52 and p65 components, as well as that of inhibitory IκB-α, were unaffected by aging. Instead we observed a marked increase in the nuclear concentration of the p52 component of the NF-κB complex, which could enhance the formation of the NF-κB complex in the nuclear fraction. A high level of the p52 component could also be observed in NF-κB-binding complexes in supershift assays. In addition to the changes in p52 protein level, changes in the binding affinities of complexes due to the phosphorylation of the p50 [26] and p65 [27] protein components could also play a role. The phosphorylation of the NF-κB components could strongly enhance the DNA-binding affinity of proteins and improve the stability of DNA–protein complexes during PAGE.

Protein p52 can form dimers with p50 and p65 components and bind to the κB sites [25]. Protein p52 does not contain the transactivation domain and hence the dimers of p50/p52 and p52/p52 are repressive to transactivation. Supakar et al. [28] have also observed a marked increase in NF-κB-binding activity in rat liver during aging. Supakar et al. [28] observed that NF-κB acts as a negative regulator for the rat androgen receptor gene in liver. They did not analyse the protein components of the NF-κB complexes. However, it seems that the increased NF-κB-binding activity during aging reflects an inhibitory activity in gene expression. The response of NF-κB to aging seems to be analogous to the down-regulation of the heat-shock response during aging [29] although this down-regulation is induced by decreasing the binding activity of heat shock factor.

Replicative senescence of human WI-38 fibroblasts is frequently used as a model of aging *in vitro* [2]. Contrary to the aging-induced up-regulation of nuclear NF-κB binding activities in tissues, we observed a marked down-regulation of NF-κB and Sp-1 transcription factors in cultured senescent human WI-38 fibroblasts. Western blot assays showed that the protein levels of p50 and p52 were down-regulated in the nuclear fraction. The cytoplasmic and nuclear levels of IκB-α were also slightly decreased in senescent fibroblasts. We also observed a significant down-regulation of NF-κB enhancer-driven CAT reporter gene in senescent WI-38 fibroblasts. It seems that replicative senescence down-regulates the NF-κB transcription factor signalling pathway in human WI-38 fibroblasts, as we have observed in UVB-induced activation of the NF-κB system (M. Helenius, unpublished work). NF-κB responses to aging are divergent in tissues *in vivo* and cultured fibroblasts *in vitro*. However, the aging responses could be repressive in the function of the NF-κB signalling system both in tissues and cultured fibroblasts. The repressive gene regulation of NF-κB could contribute to the progressive loss of physiological capacities observed during aging [1,2].

Several lines of evidence show that oxidative stress is one of the most important contributors to the aging process in animal tissues [30,31]. The redox status of cells, and especially several oxidants, can regulate the activation of the NF-κB signalling pathway by activating stress-responsive protein kinases that activate the cytoplasmic NF-κB complex [15,24]. However, the sustained activation of the stress-responsive NF-κB signalling pathway during aging seems unlikely because, for instance, the protein levels of cytoplasmic p50, p52 and IκB-α were not affected by aging in tissues. It seems that the expression of the p52 component increases during aging in tissues, because we could not see any pathological changes, such as inflammation, in liver and heart tissues of rat, which excludes the possibility of invading cells as an origin of NF-κB changes (M. Helenius, unpublished work). The expression of the p50 component of the NF-κB complex has been reported to be greatly increased in some cancers, e.g. in lung carcinoma, leaving the expression of p52 unaffected [32]. It seems that the p50 and p52 components of the NF-κB complex might have divergent functions in the regulation of the NF-κB signalling pathway.

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IV

Characterization of aging-associated upregulation of constitutive nuclear NF- kappa B binding activity

by

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Submitted for publication

Characterization of Aging-Associated Upregulation of Constitutive Nuclear NF-κB Binding Activity

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Running head title: AGING AND NF-κB REGULATION

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 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 IKK-complex kinases (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 are involved in the observed upregulation 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 upregulation 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.

INTRODUCTION

NF-κB (nuclear factor kappa B) 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 (Siebenlist *et al.*, 1994, Ghosh *et al.,* 1998). In addition, NF-κB is involved in anti-apoptotic responses (Liu *et al.*, 1996, Van Antwerp *et al.*, 1996), which are suggested to be involved in the development of age-related, deleterious diseases (Wang 1997). We have previously shown that NF-κB binding activity increases significantly with age in many mouse and rat tissues (Helenius *et al.*, 1996a, 1996b, Korhonen *et al.*, 1997). 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. The mammalian NF-κB gene family consists of five members (*p50/p105, p52/p100, p65, c-rel, and relB*) (Siebenlist *et al.*, 1994, Baldwin, 1996) all containing an about 300 amino acids long Rel Homology Domain (RHD) at their N-terminus (Siebenlist *et al.*, 1994). 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 non-covalent interactions with inhibitor proteins, IκBs (inhibitor of NF-κB) (Gilmore & Morin, 1993). 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 (6-8) of the ankyrin repeats (Bork, 1993, Nolan & Baltimore, 1992), 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 (Siebenlist *et al.*, 1994). 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 IKK-complex (IκB kinase complex) (DiDonato *et al.*, 1997, Zandi *et al.*, 1997, Mercurio *et al.*, 1997), 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 (Zandi *et al.*, 1998) triggering their ubiquitination and degradation by the 26S proteasome pathway (Palombella *et al.*, 1994). The released NF-κB then translocates to 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 (Baldwin, 1996) or some accessory factors may influence their binding to cognate DNA, such as HMG1(Y) protein (Zhang & Verdine, 1999).

Few direct upstream activators of IKK-complex have been identified. These include *e.g.* NIK (NF-κB inducing kinase) and MEKK-1 (Yin *et al.*, 1998). However, in certain activation circumstances, NF-κB activation does not involve the IKK-complex or even IκB degradation (Li & Karin, 1998, Kretz-Remy *et al.*, 1998, Imbert *et al.*, 1996, Singh *et al.*, 1996).

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 were, however, 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 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 EMSA and in Western blotting: anti-NF-κB, p65 subunit (Boehringer & Mannheim), HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (Cappel), PhosphoPlus IkB-α (Ser 32) Antibody kit (cat. 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, consensus and mutated AP-1 (Promega).

Isolation of proteins for EMSA and Western blotting

Nuclear and cytoplasmic proteins were released and purified from samples according to the modified protocol of Dignam (Dignam *et al.*, 1986) described in more detail in Helenius *et al.* (1996a).

EMSA (Electrophoretic mobility shift assay)

The double-stranded oligonucleotide probes used in EMSA were end-labelled using γ-^{32}P-ATP (Amersham) and T4-polynucleotide kinase (Promega) according to the protocol of 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 using poly dI/dC (Pharmacia). 10% Nonidet P40 (BDH) 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 Whatmann paper (3MM), and results were visualized either on

an autoradiography film (Fuji) or Storm 860 PhosphoImager (Molecular Dynamics).

The protein components in specific NF-κB complex were identified using supershift assay. After the 10 min binding reaction, a specific primary antibody against different components of the NF-κB complex was added, incubated further (30 min, 4 °C), and a normal gel retardation assay and visualization were performed (Helenius et al. 1996a). Supershift assays were also repeated with the treatment of nuclear proteins for 60 min. in ice prior to DNA binding reaction.

UV-crosslinking

UV-crosslinking technique was used to characterize the proteins which bind to the -κB binding site. Assays were performed as described earlier (Helenius et al. 1996a).

Western blot assays

Nuclear (15 μg) and cytoplasmic proteins (20μg) were resolved in 10 % SDS-PAGE gel electrophoresis and transferred to an Immobilon-P (Millipore) membrane using a semi-dry 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 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 x 5 min) and incubation with a secondary antibody, either HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG (1:2000 dilution in PBS, 5 % fat-free milk, 0.1 % Tween), for 1 h at room temperature. After washing (4 x 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). 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-labelled riboprobes were generated with Strip-EZ kit (Ambion) and used without further purification. 300 ng of rat liver mRNA were 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 (Church & Gilbert 1984) at 55-60 °C depending on the probe length and GC content. After that, the filters were rinsed once (1 x SSC) and washed (1 x SSC, 0.2% SDS, 68 °C, 30 min and 0.1 x SSC, 0.2% SDS 68 °C, 1h) and signals were visualized 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 3 most widely used "housekeeping" genes were tested. After stripping with Strip-EZ kit (Ambion) filters were consecutively reprobed with ^{32}P-labelled antisense riboprobes specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-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.

RESULTS

Constitutive increase in nuclear NF-kB binding activation with age

Aging induced a strong increase (approx. 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 (Helenius *et al.*, 1996a, 1996b, Korhonen *et al.*, 1997). Supershift assay showed that the nuclear NF-κB complex contained p50 and p65 subunits in rat liver (Fig. 2A). Shifted bands were faint probably due to the post-translational protein modifications (*see* Western results). The same subunits were also observed to form nuclear NF-κB complexes in mouse heart (Helenius *et al.*, 1996a).

UV-crosslinking technique was used to characterize proteins, which bind to NF-κB oligonucleotides. Figure 2B shows the major three bands over 66 kD size which probably represent the complexes of NF-κB proteins bound with labeled oligonucleotides. Post-translational modifications may affect the size of binding complexes (*see* Western results). Addition of unlabelled consensus oligonucleotides reduced the specific binding (Fig. 2B, lanes 3 and 4). 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 unlabelled 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 both in 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 (CIP) was not able to remove this band (data not shown). On the contrary, the level of p50 subunit was not affected by age (Fig. 3). The protein level of nuclear c-Rel showed a high inter-individual 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 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).

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, both in young and old animals.

The activity of the IKK complex is regulated, at least in part, by phosphorylation (Ling *et al.*, 1998, Malinin *et al.*, 1997, Woronicz *et al.*, 1997). The IKKγ subunits have been reported to be the regulatory component of the IKK complex connecting IKK to its upstream activators (Rothwarf *et al.*, 1998). 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 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 -β) 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

(reviewed *e.g.* by Dice 1993). Oxidative stress and changes in stress resistance have been suggested to be among the major contributors to the aging process in animal tissues (Sohal & Brunk, 1992). NF-κB is one of the key regulators of the cellular defensive response to oxidative stress in mammalian cells (Siebenlist *et al.*, 1994). In addition, NF-κB is involved in apoptotic responses in cells. Interestingly, cells are reported to become resistant to apoptosis during aging (Wang, 1995). 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 significant upregulation of constitutive nuclear NF-κB binding activity with age in several mouse and rat tissues (Helenius *et al.*, 1996a, 1996b, Korhonen *et al.*, 1997) suggesting that this increase is a general aging-associated change which occurs in cells, and hence a regulated process. 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, nuclear NF-κB binding activity increased significantly with age as observed earlier (Helenius *et al.*, 1996a, 1996b). 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 (Helenius *et al.*, 1996a). Western blot assays showed that the levels of p52 and p65 proteins were clearly increased both in 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 (Helenius *et al.*, 1996a, 1996b, Korhonen *et al.*, 1997). 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 post-translationally modified form of these proteins. Possible modifications could be phosphorylatation, ubiquitination, or acetylation. Treatment of samples with alkaline phosphatase (CIP) 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) (Zhang & Verdine, 1999), 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 which 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 (Betts & Nabel, 1996). 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, which control activation signals and nuclear translocation of NF-κB proteins, are also possibly involved in this up-regulation. 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 are 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 (Lee *et al.*, 1998), were also unaffected by age suggesting that they are not involved in the up-regulation 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, at least partly, due 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 are involved in the up-regulation of nuclear NF- κB binding activity and the increased nuclear level of p52 and p65 subunits in livers of aged animals.

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FIGURE LEGENDS

FIG. 1. Nuclear NF-κB binding activity is constitutively increased in old rat livers. (A) Old samples are marked with asterix. (B) Storm phosphoimager calculation of pixel values (mean ± SD) of specific NF-κB binding. 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 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 100x concentration of competing unlabelled 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 (lane2) liver. Lanes 3 and 4, respectively, show NF-κB DNA binding with 50x concentration of competing unlabelled NF-κB oligonucleotide. Lanes 5 and 6 show DNA binding of young and old liver samples, respectively, to the mutated NF-κB oligonucleotide binding site. Markers: *arrow* shows the specific NF-κB complex, *arrowhead* shows the supershifted complex and *star* shows an unspecific binding.

FIG. 3. Changes in protein levels of NF-κB proteins p50, p52, p65 and c-Rel during aging. The figure shows the protein levels both in nuclear and cytoplasmic fractions in the livers of young and old (lanes marked with asterix) 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. Asterix marks the liver samples from old rats. IκBα-P panel shows the positive and negative control sample on right (the antibody kit from New England BioLabs, *see* Reagents).

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

FIG 6. mRNAs 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 asterix. 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 asterix). (C) Calculation of Storm phosphoimager values (mean ± SD). The expression level of cyclophilin was used for the normalization of mRNA samples (see Methods).

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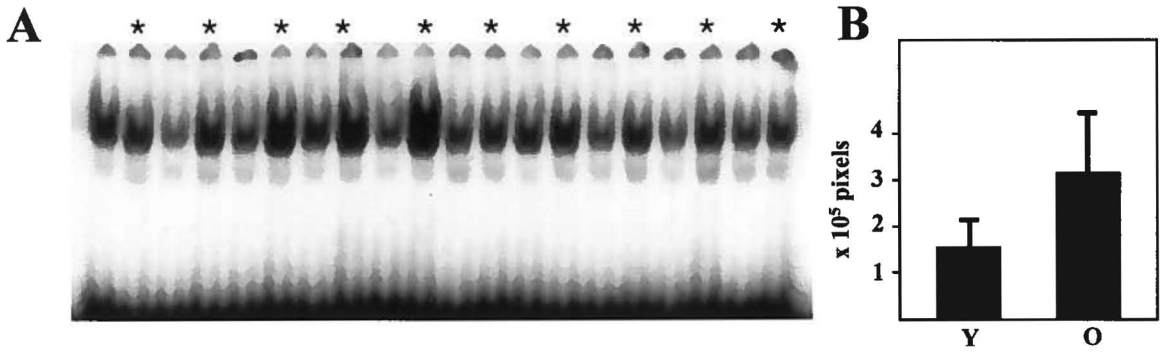

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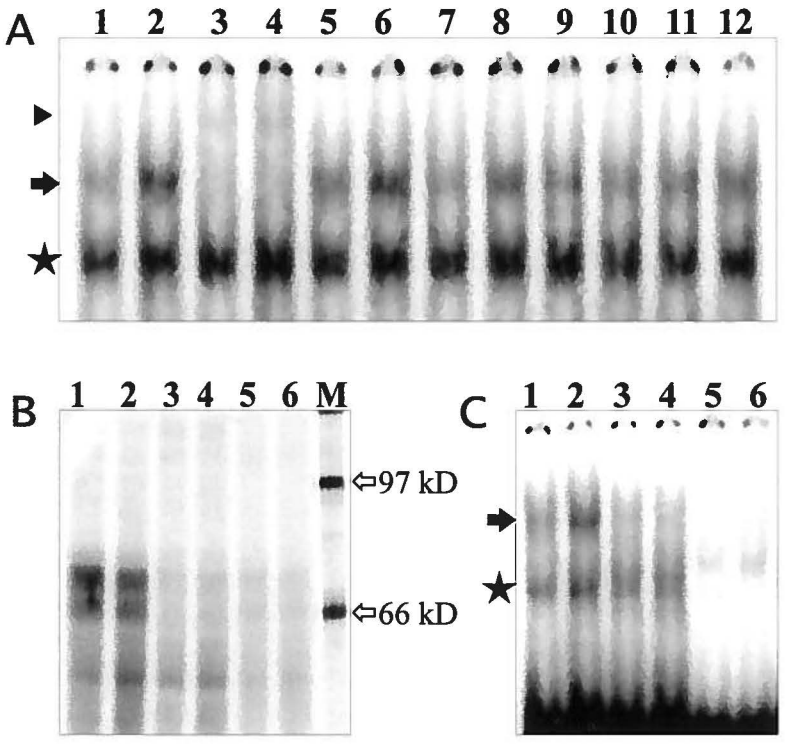

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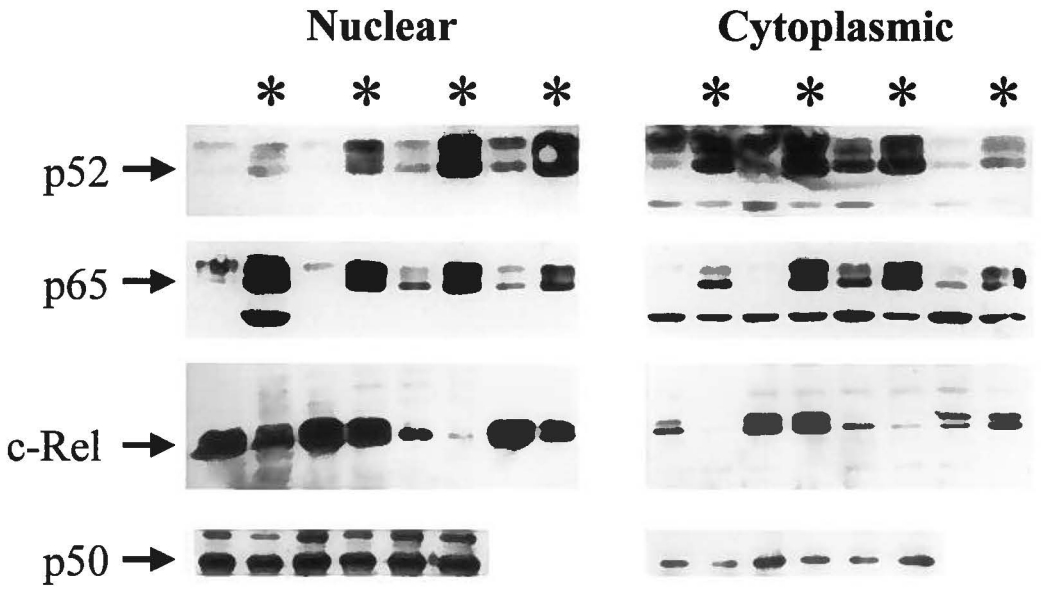

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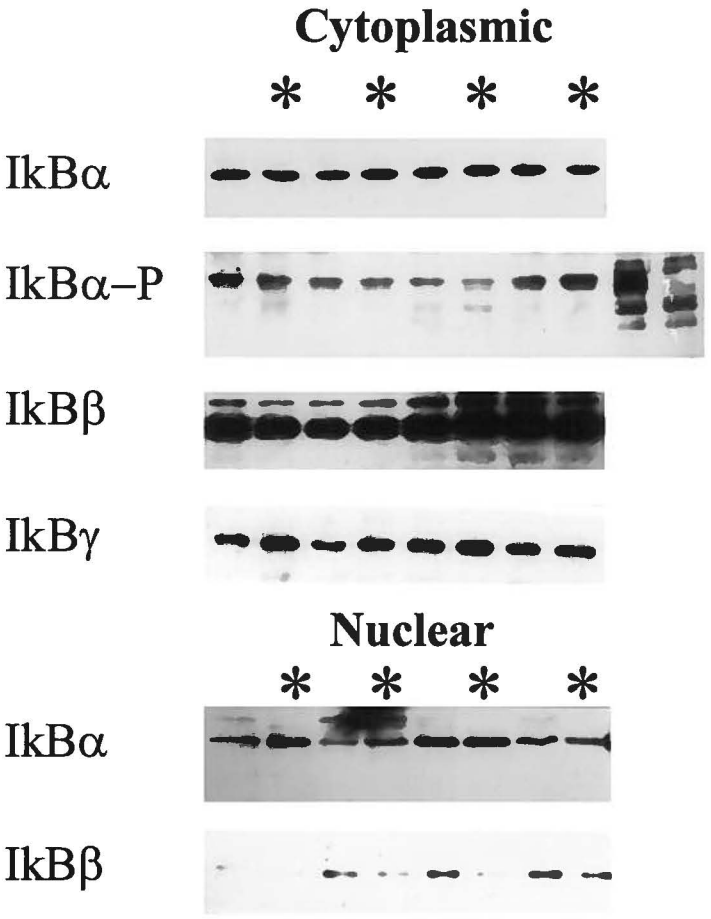

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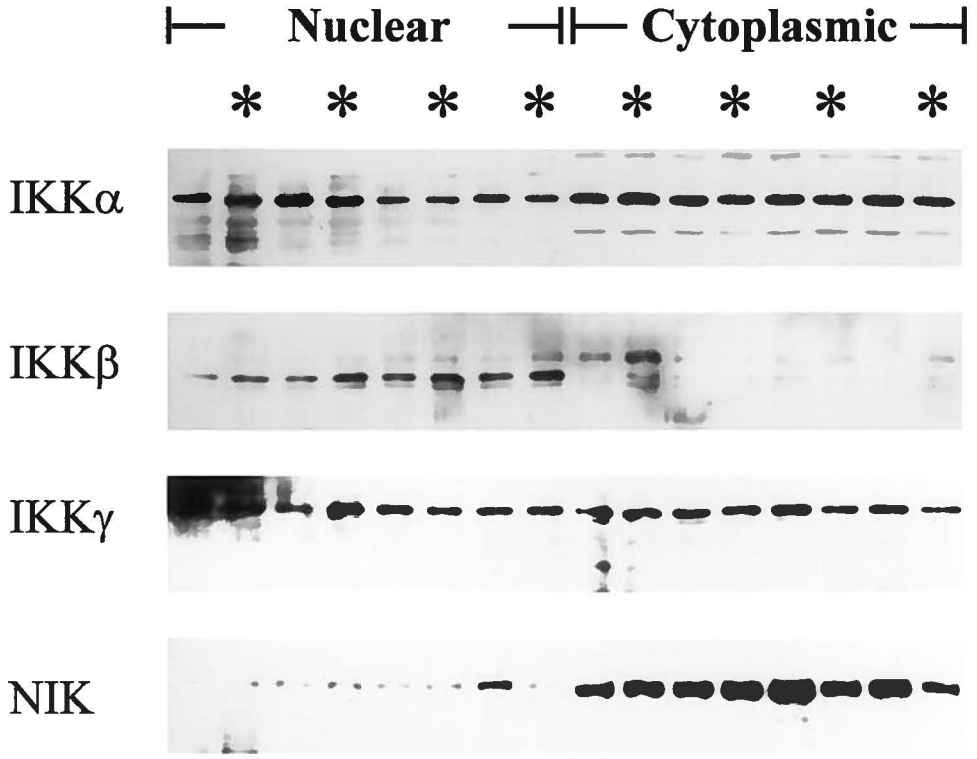

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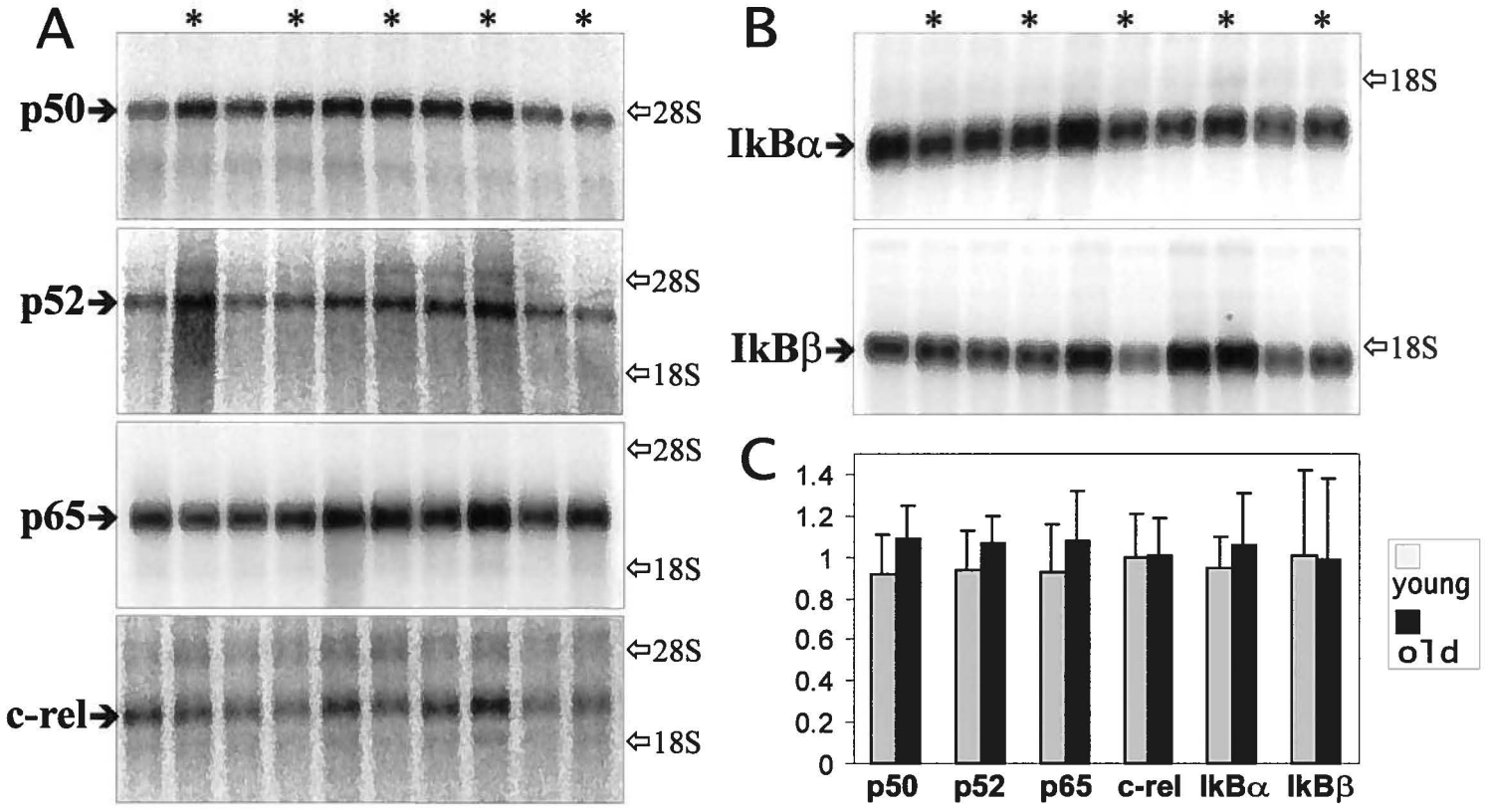
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Fig 6.

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Attenuation of NF-kappa B signalling response to UVB light during cellular senescence

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Helenius, M., Mäkeläinen, L. & Salminen, A.

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Attenuation of NF-κB Signaling Response to UVB Light during Cellular Senescence

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The ability of cells to adapt to environmental stresses undergoes a progressive reduction during aging. NF-κB-mediated signaling is a major defensive system against various environmental challenges. The aim of this study was to find out whether replicative senescence affects the response of the NF-κB signaling pathway to UVB light in human WI-38 and IMR-90 fibroblasts. The exposure of early passage fibroblasts to UVB light inhibited the proliferation and induced a flat phenotype similar to that observed in replicatively senescent fibroblasts not exposed to UVB light. The UVB radiation dose used (153 mJ/cm^2) did not induce apoptosis in either early or late passage WI-38 fibroblasts. UVB exposure induced a prominent activation of the NF-κB signaling pathway both in early and in late passage WI-38 and IMR-90 fibroblasts. Interestingly, the response to UVB light was significantly attenuated in late passage fibroblasts. This attenuation was most prominent in DNA binding activities of nuclear NF-κB complexes. Similar senescence-related attenuation was also observed in the DNA binding activities of nuclear AP-1 and Sp-1 factors after UVB treatment. Immunoblotting and -cytochemistry showed an increase in nuclear localization of p50 and p65 components of NF-κB complexes. Supershift experiments showed that the specific NF-κB complexes contain p50 and p65 protein components but not p52 and c-Rel proteins. Cytoplasmic IκBα showed a marked decrease at protein level but an increase in phosphorylation after UVB treatment. Transient transfection assays with TK5-CAT and TK10-CAT plasmids carrying NF-κB-responsive sites of the TNFα promoter were used to analyze the functional activity of the NF-κB complexes. Results showed that UVB exposure induced an increase in NF-κB-driven CAT expression both in early and in late passage fibroblasts though the response was significantly stronger in early passage fibroblasts. Our results show that the induction of NF-κB-mediated signaling by UVB light is highly attenuated in senescent fibroblasts. This attenuation may reduce the stress resistance during cellular senescence. © 1999 Academic Press

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INTRODUCTION

Several lines of research have shown that stress resistance is a major regulator of the aging process [1–3]. The ability to respond to environmental stress decreases during aging. Interestingly, the long-lived mutants of *Caenorhabditis elegans* show a stress-resistant phenotype, e.g., to environmental toxins, especially to those toxins that induce oxidative stress. Cloned gerontogenes are associated simultaneously with increased resistance to several kinds of environmental stress and life extension [1, 2].

The NF-κB signaling pathway is a key regulator of defensive responses to environmental challenges, such as radiation, oxidative stress, numerous chemical agents, injuries, viruses, and parasites [4]. This pathway has been conserved during evolution and is an ancient host defense system in organisms, such as vertebrates, insects, and plants [5]. Recently, it has been shown that NF-κB signaling induces an antiapoptotic response [6, 7]. We have previously observed that the DNA binding activities of NF-κB factors are constitutively increased in aging tissues [8]. This increase might reflect the increased stress during aging and the protective adaptive response of tissues to stress-related apoptosis and degeneration.

UVB radiation provides a good model for studying cellular stress responses and adaptation mechanisms to environmental stress [9]. UVB radiation has been shown to be an effective inducer of NF-κB signaling [e.g., 10]. The purpose of this study was to find out whether replicative senescence affects the activation of the NF-κB signaling system in human WI-38 and IMR-90 fibroblasts and whether cellular aging affects the transcriptional responses induced by NF-κB signaling. Our results show that replicative senescence attenuates the activation of the NF-κB signaling pathway. Transient transfection assays with constructs carrying NF-κB-driven reporter genes revealed that

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the functional activity of nuclear NF-κB complexes was significantly stronger in early passage than in late passage fibroblasts. These senescence-associated effects in NF-κB signaling lead to reductions in the stress resistance during aging.

EXPERIMENTAL PROCEDURES

Cell culture. Human lung WI-38 and IMR-90 fibroblasts as well as SV-40 virus-transformed subline of WI-38 were obtained from the American Type Culture Collection. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum and the antibiotics penicillin and streptomycin [8]. Fibroblasts were replicatively senesced by culturing from 15 passages up to 33–35 passages before being used in the assays. These late passage fibroblasts were replicatively senescent and their proliferative capacity was very low (data not shown).

UVB-light exposure. Fibroblasts were washed once with PBS and exposed to UVB radiation in the DMEM without phenol red and serum using four Philips TL 20W/12 lamps. This lamp emits mainly UVB, peaking at 308 nm. Radiation was carried out at room temperature at a target distance of 30 cm and normally for 3 min. After radiation, the medium used in UVB exposure was replaced with normal culture medium. The radiation dosage was 153 mJ/cm^2 for UVB at the dish level measured by a UVX radiometer provided with a UVX-31 sensor (UVP, Inc., San Gabriel, CA). Control samples were subjected to the same conditions without UVB illumination.

Electrophoretic mobility shift assay (EMSA). The EMSA method was used to analyze the DNA-binding activities of NF-κB, AP-1, and Sp-1 factors in nuclear extracts. Briefly, nuclear proteins were isolated by the modified method of Dignam *et al.* [11], as we described earlier in detail [8]. All isolation media used were supplemented with Completed proteinase inhibitors (Boehringer). Protein concentrations were assayed by the DC Protein Assay kit from the Bio-Rad Company. Consensus and mutated double-stranded oligonucleotide binding sites of NF-κB, AP-1, and Sp-1 factors were labeled with T4 polynucleotide kinase by using the oligonucleotides, labeling reagents, and protocol of Promega. Protein–DNA binding assays were performed as described earlier [8]. Briefly, 10 μg of nuclear protein was used in each reaction. Nonspecific binding was blocked by using 2 μg of poly(dI–dC)–poly(dI–dC) (Pharmacia). The binding reaction contained 4% glycerol, 1.0 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 1.0 mM DTT, and 10 mM Tris/HCl, pH 7.5. Bound and unbound probes were separated in a native 4% polyacrylamide gel with a running buffer of 50 mM Tris, 380 mM glycine, and 2 mM EDTA, pH 8.5. Autoradiography films (Reflection Nef-496; Du Pont) were exposed for 1–2 days at −80°C.

Supershift experiments. Supershift experiments were performed to show the protein components in specific NF-κB complex. Antibody treatment and gel retardation assays were performed as described earlier [12] using specific Santa Cruz antibodies against p50 (H-119X), p52 (447X and K-27X), p65 (H-286X), and c-Rel (CX and NX).

Western blot assays. The protein levels of NF-κB components and IκBα were assayed by Western blot using antibodies from Santa Cruz against p50 (NLS), p52 (K-27 and D-32), p65 (C-20), and IκBα (C-15). The level of phosphorylated IκBα was assayed using the kit from New England BioLabs. The phosphospecific antibody detects the phosphorylation of IκBα at Ser32. We have earlier described in detail the conditions for gel electrophoresis, blotting, and immunostaining of filters [8]. Results were shown by Western ECL method using Supersignal Substrate from Pierce and their protocol.

Immunocytochemistry. Fibroblasts were fixed with 100% methanol for 5 min at −20°C. Primary antibodies against p65, p50, and IκBα were from Rockland Company (Anti-NFκB/IκB kit, K-711). Rhodamine-conjugated goat anti-mouse IgG (Fc-specific) was used as secondary antibody (Cappel). Nuclear dye Hoechst 33258 (Sigma) was used to show the localization of nuclei.

Transfections. Early and late passage WI-38 fibroblasts were transiently transfected with TK5-CAT and TK10-CAT plasmids which contain the κB enhancer region of mouse TNFα gene with six (TK5) or two (TK10) NF-κB binding sites linked to the chloramphenicol acetyltransferase (CAT) reporter gene [13]. Cells were cotransfected with plasmid pCH110 (Amersham) containing the lacZ reporter gene to exclude differences in transfection efficiencies and translation capacities between different types of cells. Fibroblasts were transfected overnight with the DOTAP lipofection reagent (Boehringer). Next morning, transfection reagent was aspirated, cells were washed once, and fresh medium was added. Cells were further incubated for 3 h to overcome the stress induced by transfection. The fibroblasts were UVB radiated for 1 min (51 mJ/cm^2) as described above. Proteins were harvested 50 h after the radiation. The expression of CAT was analyzed with a CAT-ELISA kit (Boehringer) and that of β-galactosidase (lacZ) by using chlorophenol red–β-D-galactopyranoside (Boehringer) as a substrate. Assays were performed as suggested by the manufacturer.

Caspase-3 assay. Cytosolic extracts were prepared by treatment of cells with 10 volumes of ice-cold hypotonic buffer as used in EMSA assays [8]. The activities of caspase-3 were assayed using fluorogenic substrate Ac-DEVD-AMC (Pharmingen). The substrate was used at a final concentration of 20 μM. Assays were performed according to the Pharmingen protocol.

Drug treatments. The role of protein phosphorylation on the NF-κB signaling response to UVB radiation was studied using tyrosine kinase inhibitors tyrphostin AG 126 and herbimycin A, well-known inhibitors of NF-κB activation [14, 15], and protein phosphatase inhibitors okadaic acid and sodium fluoride. Okadaic acid is an inhibitor of protein phosphatase 2A and 1 [16], while sodium fluoride inhibits serine/threonine phosphatases [17]. The WI-38 fibroblasts used in this experiment were from passage 26, still retaining effective proliferation capacity. The final concentrations of these drugs were 50 μM for tyrphostin AG126, 10 nM for herbimycin A, 50 nM for okadaic acid, and 1 mM for sodium fluoride. Fibroblasts were preincubated with drugs for 3 h before UVB exposure and after radiation they were incubated for 12 h.

Statistics. Autoradiographs were analyzed with an Image Grabber 2.1 (Neotech Ltd.) and Image 1.43 programs to measure the mean densities of DNA-binding complexes. All assay results were analyzed with a univariate analysis of variance and the nonpaired Student *t* test.

RESULTS

UVB Light Induced Morphological Changes in Human WI-38 Fibroblasts

Several earlier reports [for reviews see 18, 19] have described that replicatively induced cellular senescence causes a major reduction in replication and produces a flat phenotype in human WI-38 fibroblasts. Figure 1C shows late passage flat and nonreplicative senescent fibroblasts. Interestingly, the exposure of early passage fibroblasts to UVB light also blocked the proliferation and induced a flat phenotype (Fig. 1B). The exposure of late passage fibroblasts to UVB light did not induce any prominent morphological changes in the cells (Fig. 1D). In SV-40-transformed human WI-38 fibroblasts, UVB exposure induced only slight

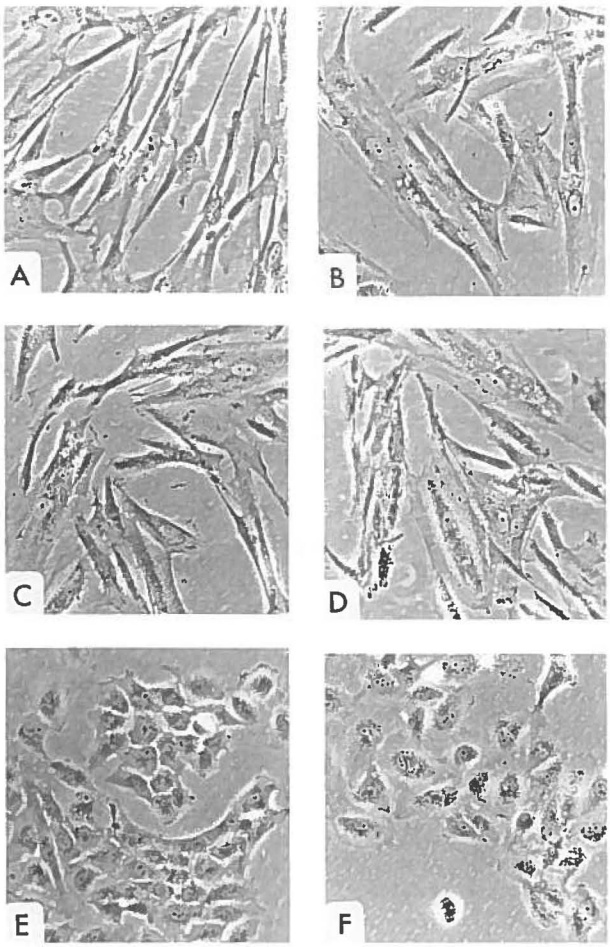

FIG. 1. Morphological changes in human WI-38 fibroblasts induced by replicative senescence and exposure to UVB light. Early passage control (A) and UVB-treated (B) cells, late passage control (C) and UVB-treated (D) cells and SV-40-transformed control (E) and UVB-treated (F) cells. Fibroblasts shown are 12 h after UVB treatment. Original magnification is 95×.

changes in phenotype (Fig. 1F). UVB treatment reduced proliferation, and apoptotic changes were induced in some SV-40-transformed fibroblasts. This was seen as nuclear fragmentation with Hoechst 33258 staining (data not shown). In UVB-treated early and late passage fibroblasts, we did not detect any apoptotic nuclei.

Since UVB treatment can induce apoptosis [20], we assayed also the activity of caspase-3 enzyme 2 days after UVB light exposure of fibroblasts. The activities of caspase-3 were slightly higher in all UVB-treated fibroblasts, but there were not statistically significant differences ($P > 0.01$) (Fig. 2). This verifies the morphological observations (see above). It seems that either the UVB dosage used in our experiments was too low to induce apoptosis [20] or WI-38 fibroblasts are resistant to UVB-induced apoptosis.

Replicative Senescence Attenuates the NF-κB Signaling Response to UVB Light

Replicative senescence of human WI-38 and IMR-90 fibroblasts induced a prominent attenuation in the NF-κB signaling response to UVB light as detected by EMSA, Western blot, and immunocytochemical techniques as well as transient transfections and reporter gene techniques. Figure 3 shows the EMSA results. The exposure to UVB light induced a major increase in nuclear DNA binding activity of NF-κB factors both in early and in late passage as well as in SV-40-transformed WI-38 fibroblasts (Fig. 3A). The appearance of the increase was somewhat slower in late passage than in early passage fibroblasts (Fig. 3A). Normally, a marked increase in the DNA binding activity was present already 6 h after UVB treatment (Figs. 3A and 3B) and a fairly intense binding activity was present up to 24 h after UVB treatment (Fig. 3B). Interestingly, the NF-κB response was markedly stronger in early passage than in late passage fibroblasts (Fig. 3B). We also observed that the UVB light response of the DNA binding activity of NF-κB in human IMR-90 fibroblasts (data not shown) was similar to that shown in Fig. 3 for human WI-38 fibroblasts. The response was also more prominent in early passage than in late passage cells.

Supershift assays were used to evaluate the components in nuclear NF-κB complexes. Figure 4 shows that the specific antibodies against p50 and p65 proteins but not those against p52 and c-Rel supershifted the specific NF-κB binding complexes in EMSA assays. The lack of the supershift with anti-p52 and anti-c-Rel was verified with two different antibodies (see Experimental Procedures) and by overexposing the gel (see Fig. 4). The specific NF-κB complexes of nontreated and UVB-treated cells were fairly equally supershifted

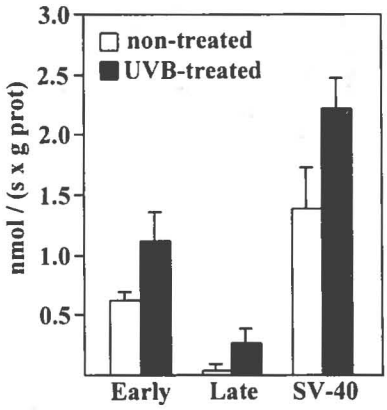

FIG. 2. The effect of UVB exposure to caspase-3 activation in early and late passage and SV-40-immortalized fibroblasts. Cells were harvested 2 days after treatment. Values are means ± SD (n = 4). Values are expressed as nmol AMC/(s × g cytoplasmic protein).

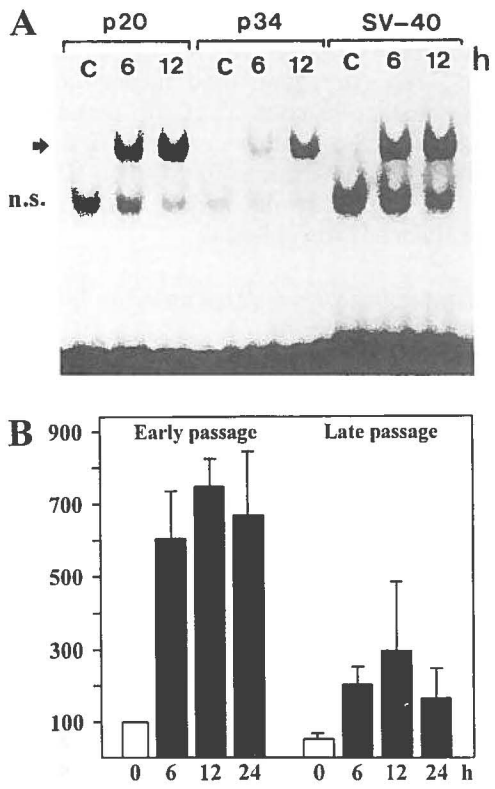

FIG. 3. Replicative senescence attenuates the UVB light-induced response in DNA binding activity of NF-κB factors. (A) A representative EMSA assay of NF-κB binding activity 6 and 12 h after UVB light exposure in early (p20) and late (p34) passage and SV-40-transformed WI-38 fibroblasts. (B) Densitometric comparison of specific NF-κB binding activities in early and late passage WI-38 fibroblasts. Columns show the average ± SD (n = 5). Values are percentage compared to nontreated samples. The binding activity of the early passage nontreated sample was calculated as 100%. Increases in NF-κB binding activities are statistically significant ($P < 0.01$) both in early and in late passage fibroblasts.

both in early and in late passage fibroblasts (Fig. 4). None of the antibodies used supershifted the nonspecific (n.s.) band (Fig. 4). It seems that the specific NF-κB complex involves p50 and p65 protein components.

We also studied whether the exposure of UVB light affects the DNA binding activities of AP-1 and Sp-1 factors (Fig. 5). Early passage WI-38 fibroblasts showed a more prominent constitutive binding activity of both factors than late passage cells (Fig. 5, lanes 1 and 2). This is in accordance with our previous observations [8]. UVB light treatment increased the DNA binding activities of AP-1 and Sp-1 in early passage fibroblasts but not in late passage fibroblasts (Fig. 5).

Western blot assays showed that the UVB light exposure slightly increased the nuclear levels of p65 and p50 proteins but not those of p52 and c-Rel (Figs. 6A and 6B). Increases in nuclear protein levels were not as prominent as the changes seen in EMSA (Fig. 3). Furthermore, differences in responses between early and late passage fibroblasts appeared only in the levels of p50 and p65 (Figs. 6A and 6B). Changes were more marked in early passage fibroblasts. Interestingly, the protein level of IκBα was clearly reduced 12 and 24 h after UVB exposure in the cytoplasm of early and late passage WI-38 fibroblasts as well as in SV-40-transformed fibroblasts (Fig. 6C). The nuclear level of IκBα was unaffected or slightly decreased after UVB radiation (Fig. 6C), which shows that there is no increase in the nuclear transportation of IκBα proteins. A decrease in cytoplasmic IκBα level may enhance the nuclear transportation of NF-κB/Rel factors [4].

Immunocytochemical staining of p65 protein was performed to study the UVB exposure-induced changes in the intracellular localization of NF-κB components.

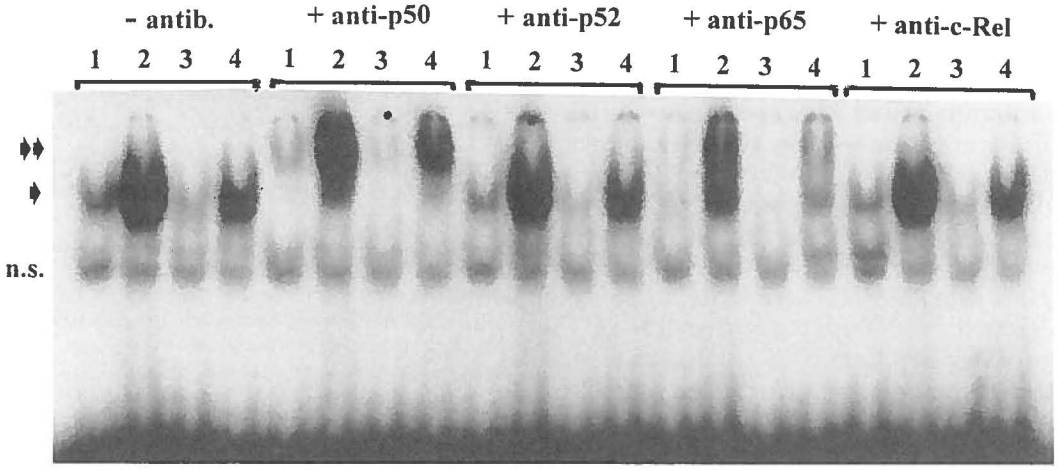

FIG. 4. The supershift of NF-κB complex with specific antibodies against p50, p52, p65, and c-Rel proteins. Lanes: (1) early passage nontreated, (2) early passage UVB-treated, (3) late passage nontreated and (4) late passage UVB-treated. The following Santa Cruz antibodies were used: p50 (H-119X), p52 (447X), p65 (H-286X), and c-Rel (CX). Specific NF-κB complex (♦), supershifted NF-κB complex (♦♦), n.s., nonspecific band.

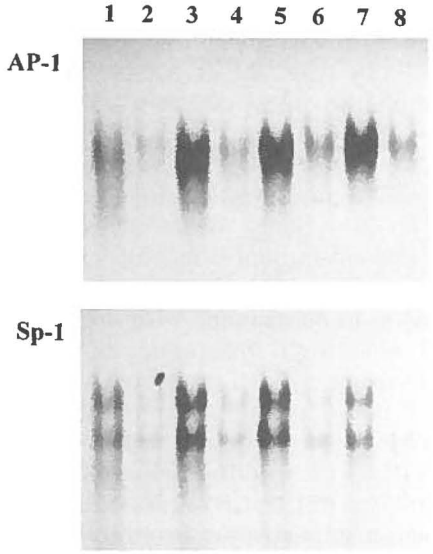

FIG. 5. UVB light induced changes in DNA binding activities of AP-1 and Sp-1 in early and late passage WI-38 fibroblasts. Lanes: Early (1) and late (2) passage nontreated, early (3) and late (4) passage 4-h posttreated samples, early (5) and late (6) passage 12-h posttreated samples, early (7) and late (8) passage 24-h posttreated samples.

Figure 7 shows that UVB exposure increased the number of fibroblasts containing the p65-positive nucleus. The response was present already 4 h after UVB treatment (Figs. 7B and 7E). The decrease in the cytoplasmic level of IκBα after UVB exposure (Fig. 6) could also be verified by immunocytochemical staining (data not shown).

We also studied whether UVB exposure affects the phosphorylation of IκBα protein. Figure 8 shows that UVB exposure induced a prominent increase in the phosphorylation level of IκBα protein at Ser32. The increase was clearly stronger in the early passage than in the late passage fibroblasts (Fig. 8). The response disappeared 45 min after the exposure (Fig. 8). We also studied whether UVB exposure affects the protein levels of the two subunits of IκB kinase complexes, IKKα and IKKβ [29]. Figure 8 shows that the protein levels of both α and β subunits decreased after UVB treatment, especially in late passage fibroblasts.

Transient transfection assays with TK5-CAT and TK10-CAT plasmids carrying the NF-κB-responsive sites of the TNFα promoter [13] were used to analyze the functional activity of NF-κB complexes after UVB induction. Figure 9 shows that UVB treatment significantly activated the expression of reporter gene CAT in both cases. The TK5-CAT construct carrying six NF-κB sites was more effective than the TK10-CAT construct with two NF-κB sites (Fig. 9). Interestingly, the UVB response of NF-κB-driven CAT expression was more potent in early passage than in late passage fibroblasts (Fig. 9). This was not due to differences in transfection or protein synthesis efficiencies because the results were compared to the expression of cotransfected lacZ reporter gene (see Experimental Procedures). These results show that the response to UVB exposure in NF-κB complexes represents the transcriptional activation, not repression, of the NF-κB signaling pathway. This response seems to be clearly attenuated in senescent fibroblasts.

Protein Kinase Inhibitors Attenuate the NF-κB Response to UVB Light

Since protein kinases regulate NF-κB signaling [21], we studied next whether the UVB light-induced NF-κB response is mediated by protein kinases. We examined the effects of two protein kinase inhibitors, tyrphostin AG 126 and herbimycin A, which inhibit NF-κB signaling in some induction models [14, 15]. We also studied

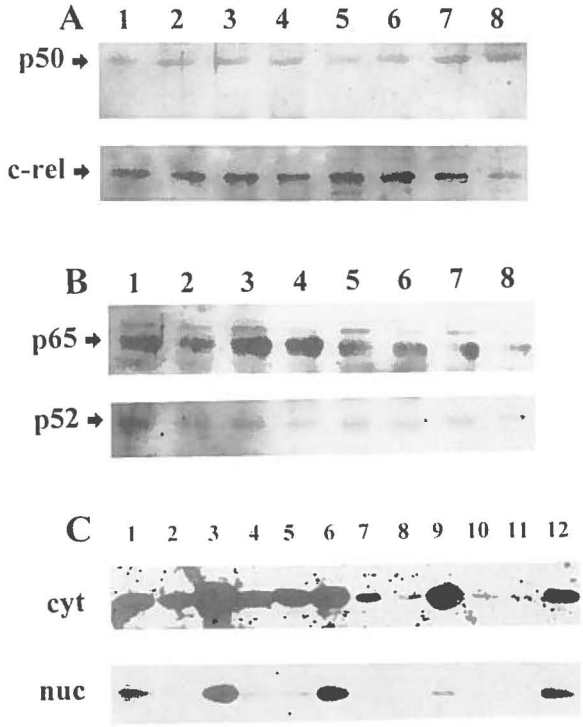

FIG. 6. UVB light induced changes in nuclear levels of p50, p52, p65, and c-Rel proteins and in cytoplasmic and nuclear levels of IκBα protein. (A) Nuclear p50 and c-Rel proteins. Lanes: Early (1) and late (5) passage nontreated cells, early (2) and late (6) passage cells 4 h after treatment, early (3) and late (7) passage cells 12 h after treatment, and early (4) and late (8) passage cells 24 h after treatment. (B) Nuclear p52 and p65 proteins. Lanes: Early (1) and late (2) passage nontreated cells, early (3) and late (4) passage cells 4 h after treatment, early (5) and late (6) passage cells 12 h after treatment, early (7) and late (8) passage cells 24 h after treatment. (C) Cytoplasmic (cyt) and nuclear (nuc) IκBα protein. Lanes: (1, 2, 3) nontreated, (4, 5, 6) 4-h posttreated, (7, 8, 9) 12-h posttreated, and (10, 11, 12) 24-h posttreated early and late passage and SV-40-transformed WI-38 fibroblasts.

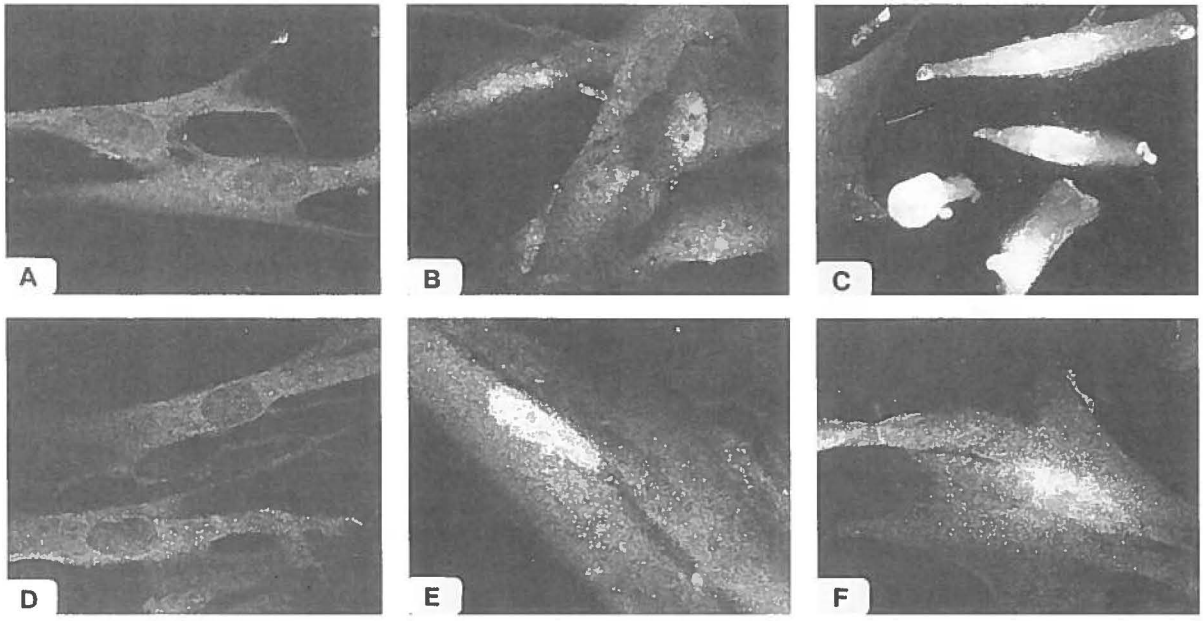

FIG. 7. Immunocytochemical localization of p65 protein in UVB exposed WI-38 fibroblasts. Shown is the immunocytochemical staining of early (A) and late (D) passage nontreated cells, early (B) and late (E) passage cells 4 h after treatment, and early (C) and late (F) passage cells 24 h after treatment. Magnification is 560×.

whether the protein phosphatase inhibitors okadaic acid and sodium fluoride would potentiate the UVB response in NF-κB activation. Results in Fig. 10 show that tyrphostin AG 126 and herbimycin A treatments strongly attenuated the UVB exposure-induced increase in DNA binding activity of NF-κB factors. Okadaic acid and sodium fluoride did not potentiate the UVB response in NF-κB binding activity (Fig. 10). However, these phosphatase inhibitors markedly increased the DNA binding activity of NF-κB in cytoplasm treated with deoxycholate (Fig. 10). Deoxycholate activates the inhibited cytoplasmic NF-κB complexes to bind DNA target sites [22]. These results show that the UVB-induced increase in DNA binding activity of NF-κB complexes is under the regulation of protein phosphorylation, and hence the attenuation of the NF-κB signaling response during cellular senescence might reflect changes in the regulation of phosphorylation cascades.

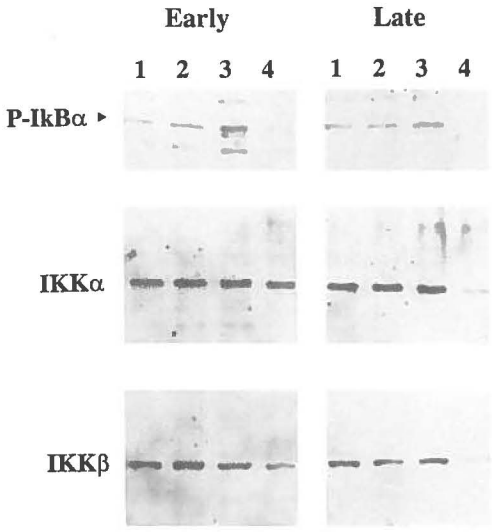

FIG. 8. UVB exposure induced changes in the phosphorylation of cytoplasmic IκBα protein (top) and the protein levels of α and β subunits of IκB kinase complex (middle and bottom). Lanes: (1) nontreated, (2) 5 min after UVB treatment, (3) 15 min after UVB treatment, and (4) 30 min after UVB treatment. Antibodies were from New England BioLabs (Phospho-IκBα kit), and IKKα (H-744) and IKKβ (H-470) were from Santa Cruz.

DISCUSSION

The data presented show that UVB light exposure causes a prominent activation of the NF-κB signaling pathway in human WI-38 and IMR-90 fibroblasts. Nuclear DNA binding activities of NF-κB factors were clearly increased between 4 and 24 h after treatment. Increased nuclear localization of protein components of NF-κB complexes could be observed with both Western blot and immunocytochemical techniques. Interestingly, the increase in the nuclear level of NF-κB components was accompanied with a decrease in the level of cytoplasmic IκBα protein. These observations are characteristic of activation of the NF-κB signaling pathway [4]. The key inducer of the activation may be

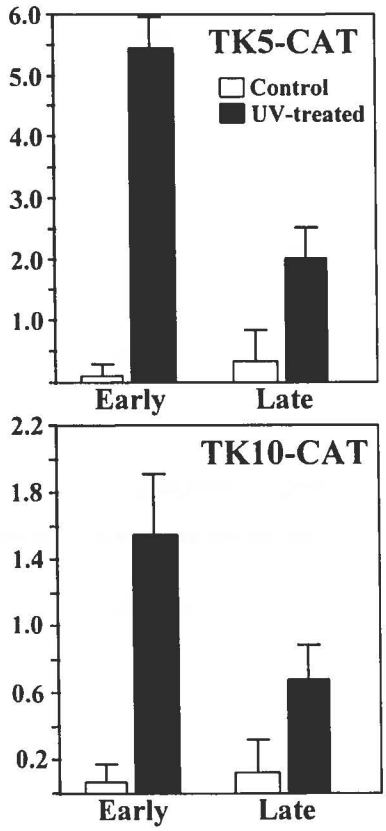

FIG. 9. UVB exposure induced expression of reporter gene CAT in early and late passage WI-38 fibroblasts transiently transfected with TK5-CAT and TK10-CAT plasmids. TK5-CAT and TK10-CAT constructs carry the NF-κB-responsive sites of the TNFα promoter (see Experimental Procedures). UVB exposure in transfection studies was for 60 s (51 mJ/cm^2). Proteins were harvested 50 h after radiation and the activity of CAT was calculated per β-galactosidase (lacZ) expression (pg CAT/mU β-gal). Values are means ± SD (n = 3 in each group). All UVB-induced responses are statistically significant ($P < 0.01$).

the phosphorylation and subsequent breakdown of IκBα protein [23].

Interestingly, we observed that the UVB radiation-induced response in NF-κB signaling was strongly attenuated in late passage human fibroblasts compared to proliferating early passage fibroblasts. The attenuation in the UVB response was most prominent in EMSA and CAT reporter gene assays. Trebilcock and Ponnappan [24] have described a similar age-related decline in the activation of the NF-κB signaling pathway in murine T cells after induction with anti-CD3. This loss in responsiveness was accompanied by a significant decline in the immune activity of T cells. However, age-related diminished responsiveness to cytokines seems to be independent of functional activity of NF-κB-mediated signaling pathway in human fibroblasts [25]. It seems that only some of the upstream signaling pathways which can activate NF-κB response show an age-related decline in cellular responsiveness to environmental stress.

Our results clearly show that the attenuation in the NF-κB signaling pathway induced by replicative senescence was due to the decline in the activation of the signaling system rather than the decline in transcriptional activity of nuclear NF-κB complexes since an equivalent decline was observed in the nuclear DNA binding activities and NF-κB-driven reporter gene expression. There are several studies to show that UVB radiation induces Ras-related signaling pathways [26] and activates c-Jun kinase [26–28]. The increase in c-Jun expression and phosphorylation can activate AP-1 transcription factor activity [26]. Increased AP-1 factor activity was also observed in this study after UVB exposure. Interestingly, late passage fibroblasts showed a clearly reduced AP-1 response after UVB radiation. This suggests that the upstream regulation of AP-1 and NF-κB signaling pathways would be mediated by the same protein kinase(s) [26]. The UVB-induced protein kinase cascade activating NF-κB may be distinct from that mediating responses of TNFα and other cytokines to NF-κB factors, since replicative senescence does not affect that signaling in human fibroblasts [25]. Recently, the IκB kinase complex with

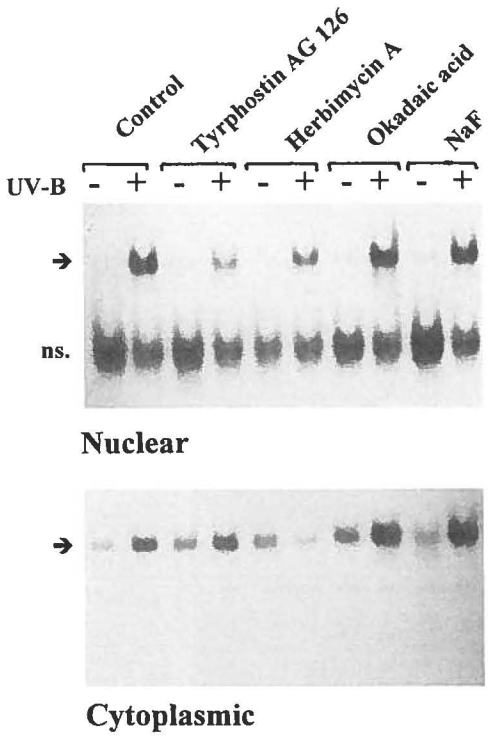

FIG. 10. Protein kinase inhibitors herbimycin A and tyrphostin AG 126 attenuate the NF-κB response to UVB light in WI-38 fibroblasts. The NF-κB binding activities (top)in nuclear fraction and (bottom) in cytoplasmic fraction induced by 2% (w/v) deoxycholate are shown. Final concentrations and treatment times are described under Experimental Procedures. ns., nonspecific binding.

IKKα and IKKβ kinases has been cloned [29] and its putative role in IκB phosphorylation has been discussed [21]. However, the roles of these kinases and other stress-related kinases in the regulation of IκB phosphorylation need to be clarified.

Several studies have shown that cellular aging reduces the activation of the MAPK cascade [30, 31]. UVB irradiation activates both p38MAPK and SAPK and both of these kinases can be inhibited by MKP-1, MAPK phosphatase [28]. The MAPK/SAPK pathway can be inhibited with herbimycin A and tyrphostin AG 126 [32, 33]. We observed that both of these protein kinase inhibitors also blocked the activation of NF-κB signaling after UVB treatment. Recent observations have shown that an increase in the expression of MKP-1 inhibits the activation of the MAPK pathway in senescent cells [30]. A defect in the activation of the MAPK/SAPK pathway may have a profound effect on the activation of transcription factors, e.g., NF-κB signaling, and subsequently on the expression of genes which maintain cellular homeostasis and stress resistance.

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